Aberrant signal transduction and protein expression in acute myeloid leukemia

Schepers, Hein

Aberrant Signal Transduction and Protein Expression in Acute Myeloid Leukemia

Hein Schepers

''They began to fulfill the destiny which was concealed in the marrow of their bones.''
Popol Vuh

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Aberrant Signal Transduction and Protein Expression in Acute Myeloid Leukemia

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Contents

Chapter 1 General introduction 7

Chapter 2 Constitutive NF-κB DNA binding activity in AML is frequently mediated by a Ras/PI3-K/PKB-dependent pathway. 41

Chapter 3 Constitutive activation of NF-κB is not sufficient to disturb normal steady-state hematopoiesis. 61

Chapter 4 Constitutive cytoplasmic localization of $p21^{Waf1/Cip1}$ affects the apoptotic process in monocytic leukemia. 67

Chapter 5 HSP27 protects AML cells against VP-16-induced apoptosis through modulation of p38 and c-Jun. 85

Chapter 6 Oncogenic Ras blocks TGF-ß-induced cell cycle arrest by degradation of p27 through a MEK/ERK/SKP2-dependent pathway. 103

Chapter 7 STAT5 is required for long-term maintenance of normal and leukemic human stem/progenitor cells. 121

Chapter 8 Re-introduction of C/EBPα in leukemic $CD34^+$ stem/progenitor cells impairs self-renewal and partially restores myelopoiesis. 139

Chapter 9 Summarizing discussion and future perspectives 159

Chapter 10 Nederlandse Samenvatting 177

References 184

List of abbreviations 194

Dankwoord 195

List of publications 199

General introduction

Chapter 1

Hematopoiesis

Hematopoiesis is considered to be a hierarchical process, with the hematopoietic stem cell (HSC) residing at the top, giving rise to all mature blood cells downstream.[1] This rare cell, which mainly resides in the bone marrow, possesses the ability to self-renew and/or undergo differentiation. Self-renewal secures the multipotent stem cell character after division of the mother cell, whereas differentiation induces changes in such a way that the progeny commits to a certain fate. This commitment can be defined as a progressive loss of differentiation potential towards other lineages.[2] A concomitant effect of increasing differentiation divisions is a decrease in the ability to self-renew.

In the past few decades, researchers have constructed a hematopoietic lineage tree (fig. 1) in which the long term-HSC (HSC^{LT}) gives rise to a short term-HSC (HSC^{ST}), followed by a multipotent progenitor (MPP).[3;4] The HSC is a relatively quiescent cell and upon differentiation towards the MPP its proliferation gradually increases from an in vivo population doubling time (77% of the cells has incorporated BrdUrd) of 17.8 days to a doubling time of 7.7 days, as estimated for murine HSCs to MPPs by means of BrdUrd incorporation studies.[5] Based on these investigations the apparent HSC cell cycle time is ~3 to 6 days,[5] which is similar to data obtained for human HSCs.[6;7] Approximately 75% of the HSC^{LT} are quiescent in G0 at any time, but are recruited regularly, so that 99% of all HSC^{LT} divide on average every 57 days.[5] These cells are, in decreasing order, able to reconstitute the entire hematopoietic system and rescue lethally-irradiated mice, confirming their multipotent and immature nature.[3;4] After this stage, a committing decision is made whether to differentiate into a common myeloid progenitor (CMP)[8] or to differentiate towards a common lymphocyte progenitor (CLP).[9] CMPs can subsequently further differentiate to granulocyte/macrophage progenitors (GMP) and megakaryocytic/erythroid progenitors (MkEP) establishing another commitment stage, whereas the CLP has to commit being a pro-B or pro-T cell. A final decisive step occurs when the GMP becomes a monocyte/macrophage or a granulocyte and the MkEP turns towards a megakaryocytic or an erythroid fate. With all bi-potential commitment decisions described above, loss of multipotentiality is increased. Recently, this classical model was challenged with data indicating that the MkEP branches off earlier at the HSCst stage, since a cell type was discovered that lacked megakaryocytic and erythroid (MkEP) potential, but retained granulocyte/macrophage (GM) and lymphoid potential.[10;11] The subsequent remaining multipotent progenitor was termed the Lymphoid primed Multipotent Progenitor (LMPP).[11] Other researchers have

postulated that the LMPP is another differentiation step downstream of the MPP and that the lack of MkEP potential was merely due to differences in the developmental time needed for different lineages to occur (see shaded areas in fig. 1).[12]

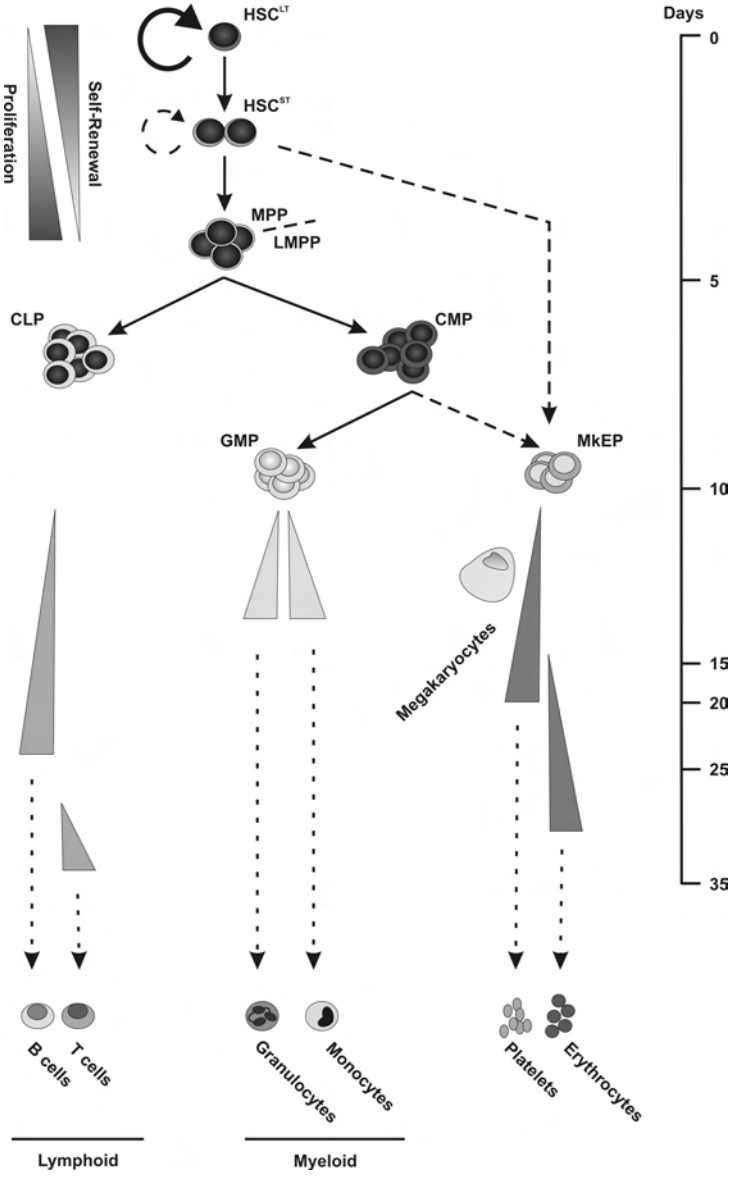

Figure 1 Hematopoiesis from Hematopoietic Stem Cell to mature blood cells.
Schematic representation of mouse and human hematopoiesis with estimated times from the start towards the peak emergence of various lineages (shaded areas). Model according to Forsberg et al,[12] Passeque et al[1] and Adolfsson et al.[11]

Regardless of the precise model, hematopoiesis provides the body with billions of mature blood cells every day (~10^{11} cells per day/adult human, ~10^{8} cells per day/adult mouse)[13] and since the right type of cell should be provided at the appropriate time with the ability to adapt to changes in steady state hematopoiesis, it is not surprisingly that hematopoiesis is a highly regulated process. Cell extrinsic mechanisms, such as hematopoietic growth factors and cell-cell interactions, and cell intrinsic mechanisms, for example transcription factor balances and epigenetic modifications, are directing hematopoiesis. Although one can argue whether ex- and intrinsic factors actually govern lineage choices[14] or are merely permissive in nature by providing a survival signal,[15] changes in signal transduction, with subsequent changes in the activity of genetic programmes, ultimately lead to a fine-tuning of processes, such as self-renewal, differentiation, proliferation and apoptosis.[1;2]

Acute myeloid Leukemia

When errors occur in the delicate balance between self renewal and differentiation, combined with disturbances in proliferation and apoptosis, a hematological disorder may develop, such as Acute Myeloid Leukemia (AML). AML is characterized by an accumulation of immature blast-like cells in the bone marrow resulting in the disruption of normal hematopoiesis.[16;17] These blast cells are of myeloid origin and are disturbed in their differentiation potential to mature cells. 9 types of AML have been classified according to the French-American-British (FAB) classification system (table 1).[18]

Table 1 French-American-British (FAB) classification of AML.

Subtype	phenotype	% of AML cases	Translocation or rearrangements	% of cases	Genes involved
M0	minimally differentiated	3%	inv(3q26) and t(3;3)	1%	*EVI-1*
M1	without maturation	15-20%			
M2	with maturation	25-30%	t(8;21); t(6;9)	40%; 1%	*AML1-ETO; DEK-CAN*
M3	promyelocytic	5-10%	t(15;17); t(11;17); t(5;17)	98%; 1%; 1%	*PML-RAR α*; PLZF-RAR *α*; NPM-RAR *α*
M4	myelomonocytic	20%	11q23; inv(3q26) and t(3;3); t(6;9)	20%; 3%; 1%	*MLL; EVI-1; DEK-CAN*
M4eo	with abnormal eosinophils	5-10%	inv(16); t(16;16)	80%	*CBF β*-MYH11
M5	monoblastic/monocytic	2-9%	11q23; t(8;16)	20%; 2%	*MLL; MOZ-CBP*
M6	erythroid	3-5%			
M7	megakaryoblastic	3-12%	t(1;21)	5%	Unknown

Figure 2 depicts myeloid differentiation downstream of the common myeloid progenitor. The gray lines indicate inherent blockades of differentiation with the immature FAB M0 with minimal differentiation at the level of the CMP and the FAB M1 direct downstream of the CMP. The myeloid AMLs M2 and M3 show undifferentiated cells of the granulocytic lineage, M4 and M5 are immature monocytic blasts, while M6 and M7 respectively designate immature cells of erythroid or megakaryocytic lineage.[18;19] The relative occurrence of these AML subtypes is shown in table 1.

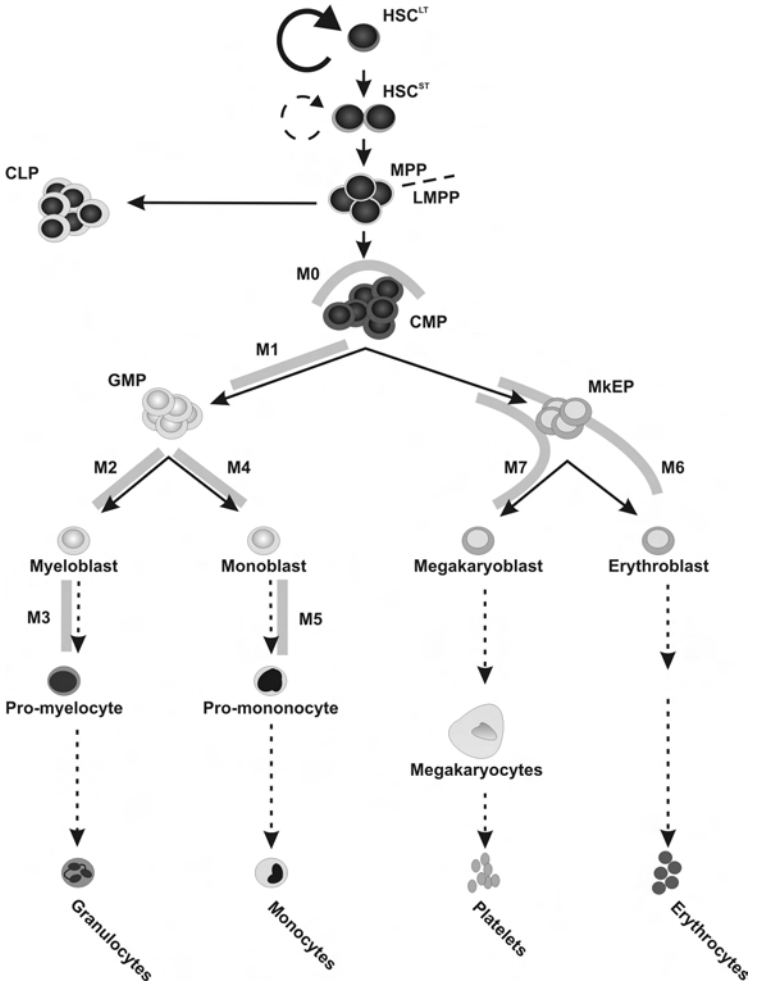

Figure 2 Overview of differentiation blocks in Acute Myeloid Leukemia.
Grey areas indicate the differentiation stages at which various AML subclassifications are blocked in their development according to the French-American-British subclassification, based on morphological appearance and histochemical staining.[18]

The distinction in AML subtypes is based on morphological appearance of the blasts combined with various histochemical stains, but does not always reflect the genetic diversity of the disease.[20;21] With the emergence of molecular genetic methods it has been possible to correlate chromosomal aberrations to specific FAB subtypes (table 1). Although such chromosomal and morphological features demonstrate the heterogeneity of AML, they are also used (amongst other parameters) to predict the response to chemotherapy, in which these chromosomal abnormalities appear to be a more important prognostic factor than the FAB subtype.[20] This has allowed the identification of

three prognostic risk groups in AML: Good, Intermediate and Poor risk.[20] The good prognostic subgroup is identified by the presence of t(15;17), t(8;21) or inv(16) abnormalities and these (younger) patients have higher rates of complete remission (CR) and a lower risk of relapse.[20;22;23] In the poor risk group, more than two chromosomal abnormalities are frequently observed or monosomies of chromosome 5 or 7 and deletions/ abnormalities of the long arm of chromosomes 5 or 3.[20;22;23] This is more common amongst older patients or patients with secondary AML. The third, intermediate, risk group has leukemic blasts with either a normal karyotype or abnormalities that are not described in the other two groups. Recently, the World Health Organization (WHO) has proposed a new classification system in which genetic and clinical features of AML are combined with immunophenotypic and biological criteria of the FAB classification.[21;24] Again three subgroups are identified: 1) AML with recurrent genetic abnormalities, 2) AML with multilineage dysplasia and 3) AML and Myelo Dysplastic Syndrome (MDS), therapy related. Of these, subgroup 1 recognizes the earlier mentioned good risk group, with the addition of AMLs with t(16;16) translocations.[21;24] If AMLs cannot be classified in one of these three groups (table 2), a fourth group (resembling the FAB classification) is used to categorize these AMLs.[21;24]

Table 2 World Health Organization (WHO) classification of AML.

Group	Description		
1	**AML with recurrent genetic abnormalities**	**Translocation or rearrangements**	**Genes involved**
	- AML with:	t(8;21) (q22;q22)	*AML1-ETO*
	- AML with abnormal BM eosinophils and:	inv(16) (p13q22) or t(16;16) (p13;q22)	*CBF β*-MYH11
	- APML with:	t(15;17) (q22;q12)	*PML-RAR α*; PLZF-RAR *α*; NPM-RAR *α*
	- AML with:	11q23	*MLL*
2	**AML with multilineage dysplasia**		
	- Following MDS or MDS/MPD		
	- Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of the cells in 2 or more myeloid lineages		
3	**AML and MDS, therapy related**		
	- Alkylating agent/radiation-related type		
	- Topoisomerase II inhibitor-related type		
	- Others		
4	**AML not otherwise categorized**	**FAB**	
	- AML, minimally differentiated	M0	
	- AML without maturation	M1	
	- AML with maturation	M2	
	- Acute myelomonocytic leukemia	M4	
	- Acute monoblastic/monocytic leukemia	M5	
	- Acute erythroid leukemia	M6	
	- Acute megakaryoblastic leukemia	M7	
	- Acute basophilic leukemia		
	- Acute panmyelosis with myelofibrosis		
	- Myeloid sarcoma		

Self-renewal and leukemia

Where normal hematopoiesis is demonstrated to be a polyclonal process, e.g. multiple HCSs contribute to hematopoiesis,[25-27] AML has been described to be a clonal disorder.[17;28] This would logically follow from the acquisition of genetic alterations, providing the affected cell with an advantage in terms of self-renewal, cell growth and survival. The normal hematopoietic system carefully balances self-renewal in the stem cell compartment and differentiation towards more committed progenitors. This differentiation is accompanied by an increase in proliferation,[5] as the HSC has been demonstrated to be relatively quiescent. This quiescence has been postulated to prevent exhaustion of the HSC pool during the life time of the host[29] and might be evaded by increasing self renewal divisions as opposed to differentiation divisions.[30;31] For AML cells it has been shown that a rare cell exists, termed the leukemic stem cell (LSC), which has extensive self-renewal capacity,[32;33] thereby giving it an advantage over normal HSCs. Some proteins have been shown to enhance and control self-renewal of hematopoietic stem cells, such as BMI-1,[34;35] STAT5,[36;37] and various HOX genes,[38] which have been shown to be deregulated in leukemic cells as well.[38-40]

Growth control and leukemia

Hematopoietic cell growth and cell cycle regulation is balanced by positive and negative growth factors. The HSC is kept in a quiescent state by growth factors, such as Transforming Growth Factor-β (TGF-β), which is likely to be the most potent negative regulator of hematopoiesis, to prevent excessive cycling and exhaustion of the system.[41;42] Positive growth factors such as Granulocyte Macrophage-Colony stimulating factor (GM-CSF) and Interleukin-3 (IL-3), not only induce differentiation, but also enhance cell-cycle kinetics within progenitor cells, subsequently leading to an expanded pool of mature cells.[43] It was demonstrated years ago that growth of clonogenic AML cells can be stimulated with colony stimulating factors, such as Granulocyte-Stimulating Factor (G-CSF) or GM-CSF.[44] Immature blasts not only respond to exogenously delivered growth factors, but have also been shown to produce growth factors themselves, such as Interleukin-1 (IL-1),[45;46] Interleukin-6 (IL-6),[47] GM-CSF,[48] G-CSF[48] and Macrophage-Colony Stimulating Factor (M-CSF),[49] which can stimulate autonomous growth in a paracrine or autocrine manner. IL-1 has been shown to upregulate autocrine production of GM-CSF by AML blasts,[50] can alleviate TGF-β-mediated reduction of colony formation[51] or can induce endothelial cells to secrete G-CSF and GM-CSF,[45] creating an amplifying loop. The autocrine production of growth factors has been linked to

in vitro cell-cycle activation of quiescent leukemic progenitor cells[52] and the autonomous in vitro growth of AML blasts has been associated with a poorer prognosis of AML patients.[53]

Besides activation of the cell-cycle by growth promoting growth factors, escape from negative growth control may also occur. In normal hematopoiesis, primitive stem/progenitor cells are kept quiescent by inhibitory growth factors and cell-cell interactions with the bone marrow micro-environment[54;55] and relief of these environmental constraints may enhance their cycling potential. TGF-β is known as a potent inhibitor of hematopoietic cell-cycle progression[41] and in various studies resistance of leukemic cells to TGF-β has been observed.[56-60] Whereas cell-cycle progression in normal hematopoietic cells is regulated by a balance of positive and negative growth factors, combined with interactions of stem and progenitor cells with bone marrow stromal cells,[55;61;62] these processes might be disturbed in leukemia leading to a growth advantage for leukemic cells.

Apoptosis and leukemia

Besides through self-renewal, differentiation and cell-cycle progression, hematopoiesis is also regulated by programmed cell death or apoptosis. The signal transduction pathways downstream of hematopoietic growth factors not only promote cell-cycle progression in target cells, but also produce a survival signal for these cells by upregulating anti-apoptotic genes or downregulating apoptosis-promoting genes.[63] Many leukemia-targeting strategies are based on the principle of inducing apoptosis, but the growth advantage of the leukemic population is partly linked to the constitutive activation of intracellular proteins that trigger the function of anti-apoptotic proteins.[40;64] This activation of anti-apoptotic proteins and the absence of pro-apoptotic proteins can hence lead to resistance of the leukemic cell to chemotherapy and has been correlated with poor diagnosis and clinical outcome.[65-67] The relapse of AML after intensive chemotherapy may be caused by resistance of a single leukemic cell towards apoptotic stimuli.

Not only autocrine production of growth factors leads to a disbalance in pro- and anti-apoptotic factors, but also the expression of oncogenes, such as Ras, or the absence of tumor supressors, such as PTEN can lead to activated signal transduction pathways, the subsequent survival of cells and contribute to leukemia.[68-71]

Differentiation and leukemia

Although the above described examples suggest that one single event is enough to cause leukemic transformation, the development of cancers and leukemias is often being viewed as a succession of multiple events contributing to the neoplastic clone.[72] In such a multiple hit model, mutations responsible for an enhanced self-renewal and block in differentiation are combined with mutations causing a proliferative or survival advantage (table 3).[73;74] This is evidenced for example by overexpression of the AML-ETO translocation, which is frequently observed in FAB M2 subclassifications. Overexpression of AML-ETO can induce a self renewal phenotype,[75] but only gives rise to an AML when combined with additional mutations.[76;77] Furthermore, AML patients in remission have indicated the presence of normal cells, but with a relation to the original leukemic clone (the original N-Ras mutation was detected in remission samples),[78] which indicated that the single alterations observed in both the AML and normal cells are not the sole cause of the emerging leukemia.

Table 3 Translocations and genes mutated in AML.

Group	Affecting	Genes involved	Fusion Partner	% of AML cases
1	Self-renewal	*AML1*		2%-3%
	Differentiation	*C/EBPa*		10%-20%
		PU.1		7%
		Fusion/translocations		
		AML1	*ETO*	See Table 1
		MLL	*ENL, AF9, AF4, CBP*	See Table 1
		CBF β	MYH11	See Table 1
		RAR α	PML, NPM, PLZF	See Table 1
		NUP98	*HOX*	1%
2	Proliferation	*N-Ras, K-Ras*		10%-20%
	Survival	*Flt3 (ITD)*		20%-35%
		c-Kit		5%-12%
		NPM		35%-60%
		PTPN11		4%

The events that underlie changes in self-renewal and differentiation often affect transcription factors, which play important roles in normal hematopoiesis as well. The transcription factor AML1 for example, is involved in the emergence of definitive HSCs[79] and its disruption will lead to a failure in development of all blood lineages. As can be seen in table 1 and 3, the transcription factor AML1 is found mutated in rare cases of AML, but is present in approximately 40% of AML FAB M2 subclassifications, as a fusion partner of ETO, resulting from the t(8;21) translocation. This translocation product has turned the transcriptional activator AML1, into a transcriptional repressor by

recruiting various co-repressors via the ETO fusion.[73;74;80] Amongst the genes repressed by AML-ETO are C/EBPα and PU.1.[80-82]

The transcription factor C/EBPα is involved in lineage commitment along the HSC-CMP-GMP route, since knock-out mice have an effect on HSC function and lack granulocyte maturation, whereas overexpression support eosinophyl and blocks monocytic lineage commitment.[2;83;84] In approximately 20% of the AML cases mutations are detected, whereas in other cases C/EBPα is downregulated or otherwise functionally impaired in its function as lineage specific transcription factor.[74;80;84;85] The transcription factor PU.1 is necessary in both MPP to CMP differentiation, as well as in lineage commitment via the CLP stage. The exact phenotype seems to depend upon the level of PU.1.[2;83;84;86] Although mutations of PU.1 are found in approximately 7% of the AML cases, it has been described to be inactivated by AML-ETO.[74;82] Table 1 and 3 further indicate the presence of various translocation products in AML. Of these, CBFβ-MYH11 has been shown to bind and repress AML1 and C/EBPα,[74;80] RARα fusion proteins have been shown to recruit transcriptional co-repressors, which might repress PU.1 and C/EBPα,[87;88] MLL fusion proteins have been demonstrated to bind to promoters and subsequently upregulate HOX genes, involved in self-renewal and lastly, NUP98-HOX fusions have been implicated in self-renewal as well.[73;74;80]

The induced mild differentiation block by this first group of mutations (or fusion oncogenes) is required, but not sufficient to induce a myeloid leukemia. The concomitant enhanced self-renewal however, prevents loss of the clone due to differentiation.[74] Secondary mutations are necessary for progression into AML. This second group of mutations, those that influence the proliferation and/or survival of hematopoietic cells, lead to the activation of receptor tyrosine kinase signalling, for example due to activated Ras,[89;90] Flt3,[91;92] c-Kit,[93] or an inactivated SHP-2 protein (PTPN11).[94;95] For these it has been demonstrated to enhance proliferation or survival, due to signal transduction via the Ras-Raf-MEK-ERK, the Pi3K-PKB and the STAT signal transduction pathways (For details, see reviews).[73;74;80] Table 3 further indicates a large cohort of AMLs with mutations in the Nucleophosmin (NPM)[96;97] gene, which has functions in stress responses, maintenance of genomic stability, regulation of transcription and various other processes, but its precise role in leukemogenesis needs to be defined.[97] In some cases, these mutations are cooperating with alterations from the first group in enhancing self-renewal, or down-regulating C/EBPα and PU.1 and hence block differentiation.[73;74;77;80;95;98;99]

Processes such as self-renewal vs. differentiation, proliferation vs. quiescence and apoptosis vs. survival are all implicated in hematopoiesis and complex regulations between these processes is observed. Stimulation of the HSC with a certain growth factor will lead to activation of a downstream signal transduction cascade, thereby not only amplifying the signal, but also expanding the downstream possibilities. Figure 3A is a schematic overview of what may happen during the life of a hematopoietic cell.

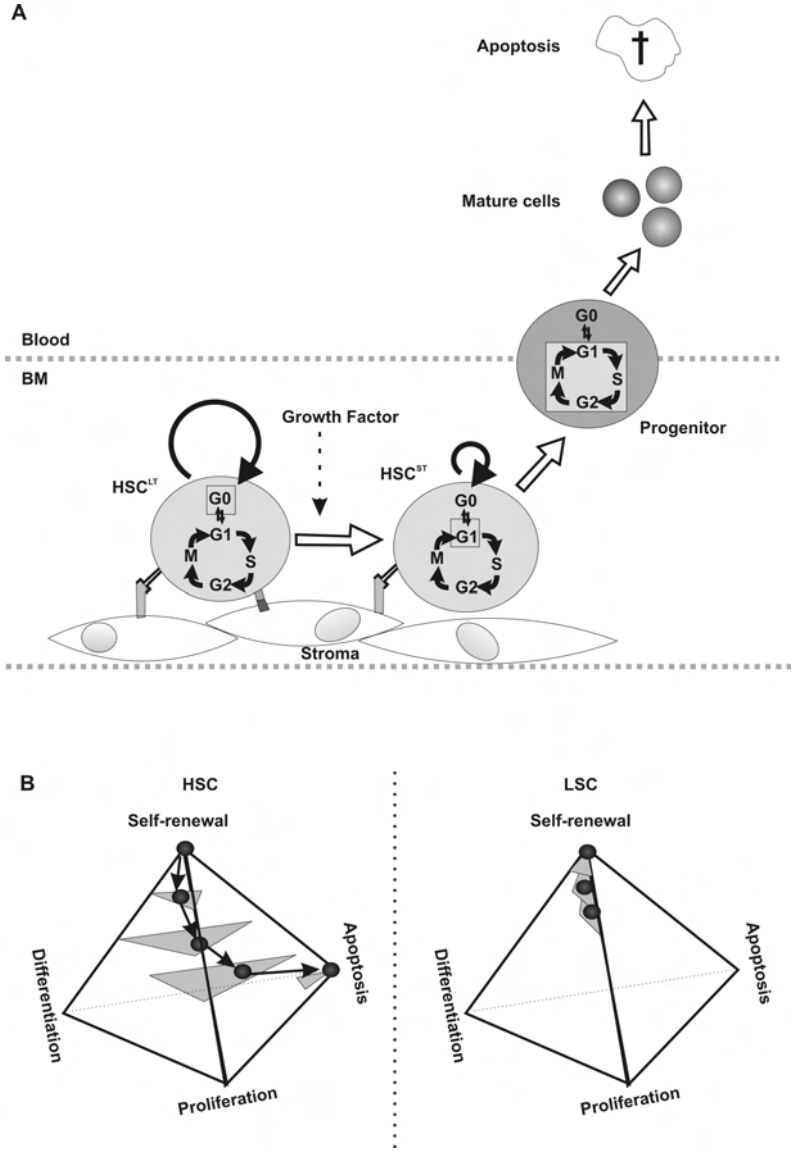

Figure 3 Hematopoiesis is a balance between intrinsic and extrinsic factors.
(A) Schematic representation of interactions involved in hematopoiesis. Direct interactions with bone marrow (BM) stroma, cell-cycle status, differentiation stage and live/dead (†) cells all influence various cell types during the lifespan of a cell. Although not shown for simplicity, most cell types produce growth factors and hence modulate the responses of other cells. (B) Hematopoiesis is shown as a balance between self-renewal, differentiation, proliferation and apoptosis, in both normal and leukemic Hematopoietic Stem Cells (HSC and LSC respectively). Grey areas indicate which processes influence a cell at any given moment during its lifetime.

The quiescent, self-renewing HSC^{LT} is residing in the bone marrow in close contact with bone marrow stroma. It is thought that this bone marrow niche regulates the stem cell property of these HSCs.[100;101] Stimulating them for example with GM-CSF or G-CSF, not only awakens it from its quiescent state to start proliferating (fig. 3A, G0→G1→S-G2-M, white arrows), but also downregulates the self-renewal program (fig. 3A, black arrows), while the differentiation program is initiated (fig. 3A, HSC^{LT} → HSC^{ST} → Progenitor → Mature cell). The interaction with the bone marrow stroma might change upon differentiation and progenitors mobilize to the peripheral blood. During the course of hematopoiesis, anti-apoptotic proteins are upregulated, growth factors are produced and excreted, whereas at the end of its lifespan, the cell is programmed to die in an orderly fashion. The growth factor-induced signal transduction pathway therefore has multiple downstream events and the outcome may depend on cell type, timing, place and combinations of the signals (fig. 3A and B). The differentiating cell may therefore be situated anywhere in the shaded triangle during its lifespan, balanced by differentiation, proliferation and apoptosis (fig. 3B, left). In comparison, AML cells, which may be affected at different stages, may react in a different way to a specific stimulus as compared to the normal counterpart. The AML “stem cell” (LSC) is blocked at certain stages of differentiation, is escaping negative growth regulation and is unresponsive to apoptotic stimuli (fig. 3B, right). Furthermore, the LSC has self-renewal potential, through which is can propagate the disease. The LSC is therefore situated in a smaller space skewed away from apoptosis and differentiation, and more around self-renewal and proliferation.

Leukemic “stem” or “progenitor” cells

The term leukemic stem cell (LSC) suggests that the transformed cell that gives rise to the clonal leukemia is an HSC. The LSC and the HSC have indeed in common that they are clonogenic cells, both of them capable of self-renewal. Phenotypic analysis of transplantable cells in immune-deficient mice have indicated that the LSC is found in the rare population of CD34+ CD38- cells, a phenotype shared with HSCs.[32;33;102-104] Since HSCs already have the

self-renewal property activated, the combination with their long life span would render them the ideal target for acquiring more mutations. This in contrast to a progenitor, which has to gain self-renewal properties and has a relatively short life span.[1] Although the CD34+ CD38- HSC is found in many leukemias to possess the leukemia initiating capacity, some exceptions have suggested that multipotent progenitors also may be the target cell. In the case of the AML FAB M3 subtype, the transforming PML-RARα translocation is exclusively found in the CD34+ CD38+ progenitor cell population and not in the CD34+ CD38- cells.[105] More recently, it was demonstrated that transformation of a Granulocyte-Macrophage Progenitor (GMP) with the MLL-AF9 fusion protein gave rise to leukemias, indicating that progenitors can also be the leukemia initiating cells.[106] Interestingly, while gene expression profiling indicated that the induced leukemia contained the progenitor gene signature from which it was derived, it also demonstrated that the self-renewal program, found in HSCs, was reactivated, since a subset of “stemcell-genes” was re-expressed. Thus, whether the self-renewal property is due to the fact that the transformed cell is an HSC, which retained its self-renewal property during partial differentiation, or that it is a progenitor, with a re-gained self-renewal property, appears to be dependent on the transforming events.

Besides their clonogenic origin, it has become clear that even within the leukemic clone heterogeneity exists.[102-104] Since a large proportion of the leukemic blasts has a limited proliferative capacity, an apparent contradiction is observed: How can the observation of clonogenic cells in a cell cycle arrest be reconciled with the appearance of growth advantage? Serial transplantation experiments in mice have shown that individual LSCs have different self-renewal capacities, giving rise to long-term and short-term LSCs, analogous to the normal hematopoietic hierarchy.[33] This suggests that the target cell for leukemic transformation is an HSC and that the normal hematopoietic program is not entirely abrogated. The actual leukemia is maintained by a rare LSC with higher self-renewal capacity than a normal HSC, but subsequent leukemic progenitors are partially blocked in their differentiation and proliferation potential, giving rise to the majority of blast cells observed in AML patients.

Together, these examples demonstrate the complexity of acute myeloid leukemia as a “stem cell” disorder with aberrant self-renewal potential, a lack of responses to apoptotic signals, inherent blockades at various stages of differentiation and altered growth properties.

Signal transduction pathways in hematopoiesis and leukemia

Apoptosis

When cells are exposed to stress, for example nutrient withdrawal, UV-radiation or chemicals, signals are elicited that either mediate survival or signal the cell to undergo a form of programmed cell death, called apoptosis. Sending two opposite signals in response to one stimulus, ensures some safeguard against derailment of this important process, since tilting to either side can result in aberrant cellular survival or in inappropriate cell death. The process of apoptosis is an essential part of tissue homeostasis in various processes, including development.[107] Induction of apoptosis can occur via an intrinsic as well as an extrinsic signal transduction pathway. The extrinsic apoptosis pathway is activated by binding of a family of related ligands, called the TNF-family, to death receptors (TNF-R family) on the cell surface. The ligand family is comprised of 5 family members, called: TNFα, lymphotoxin α (LTα), Fas-Ligand (also called CD95-Ligand or APO-1-Ligand), APO-3 ligand and TNF-Related Apoptosis-Inducing Ligand (TRAIL),[108] whereas the receptor family includes a number of members named: TNF-RI, TNF-RII, Fas, death receptor (DR) 4, DR5 and DR6.[108] Binding of the ligands, for example TNFα or Fas-L, to their cognate receptors results in trimerisation of the receptors and subsequent recruitment of the adapter molecule FADD. This adaptor molecule then interacts with the apoptosis initiating protein, procaspase-8, forming the death inducing signalling complex (DISC, figure 4).[107-109] Proteolytic cleavage of procaspase-8 into caspase-8 subsequently activates this protein and induces activation of downstream effector caspases, such as caspase 3. These will cleave specific substrates and eventually, the apoptotic pathway will result in cell shrinkage, membrane blebbing, DNA fragmentation and chromatin condensation (fig. 4). Lastly, the apoptotic cell will be removed through phagocytosis by macrophages or neighboring cells.[107;108;110]

Another apoptosis signal transduction pathway downstream from FAS, but independent of FADD is via the recruitment of DAXX to the cytosolic end of the CD95/Fas receptor.[111;112] DAXX then binds Apoptosis Signal Regulating Kinase-1 (ASK1),[109] a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, which in turn activates c-Jun N-terminal Kinase (JNK) and p38, leading to cytochrome c release and activation of caspases (fig. 4).[113-116]

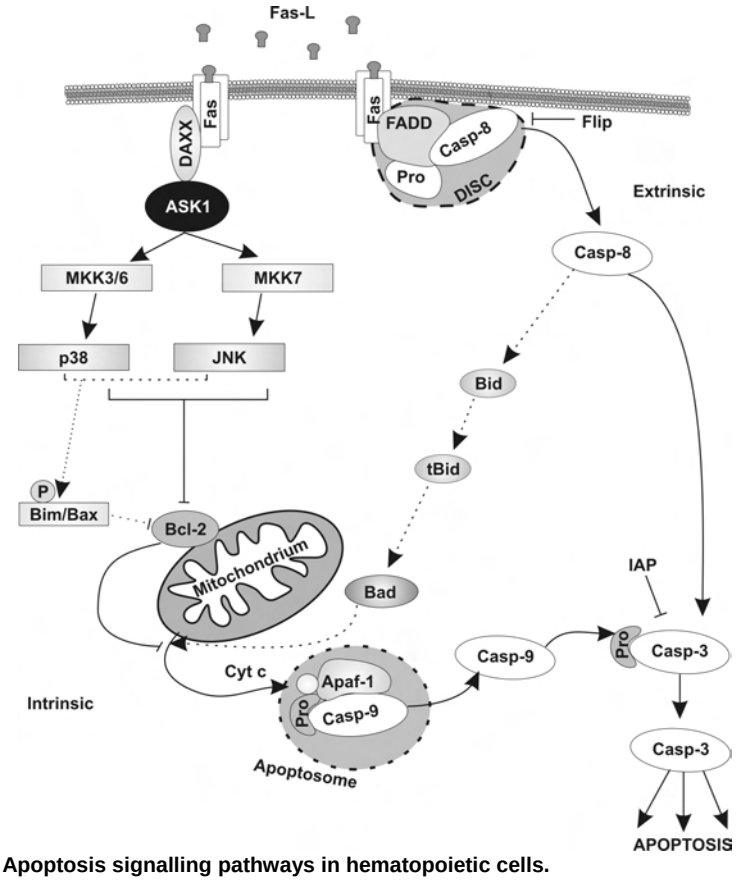

Figure 4 Apoptosis signalling pathways in hematopoietic cells.
Schematic representation of the intrinsic and extrinsic apoptosis pathways and their connection at the mitochondria.

The intrinsic pathway of apoptosis is initiated by various apoptotic stimuli and converges at the mitochondria. Various anti-cancer drugs and cellular stresses induce mitochondrial outer membrane permeabilisation (MOMP), which will lead to the release of cytochrome c from mitochondria. This cytochrome c subsequently interacts with Apoptosis Protease Activating Factor-1 (Apaf-1), which oligomerises and binds to pro-caspase-9.[117] The formation of this caspase-activating complex, termed the apoptosome, results in the activation of caspase-9, which in turn triggers the proteolytic cleavage of pro-caspase-3, leading to apoptosis (fig. 4).[117] Members of the Bcl-2 family, play an important role in regulating the mitochondrial-mediated apoptosis pathways. Anti-apoptotic family members, such as Bcl-2 and Bcl-XL and pro-apoptotic members, such as Bad, Bax, Bim and Bid, translocate to the mitochondrial membrane and modulate the permeabilization of the inner and outer membranes. Through physical interaction between pro and anti-apoptotic

family members the balance is shifted and cytochrome c release is controlled, either leading to survival or to apoptosis (fig. 4).[107;108;110] The extrinsic and intrinsic apoptosis pathways also interconnect at the mitochondria. The above mentioned DAXX-ASK1-mediated JNK activation can lead to an inhibition of the anti-apoptotic Bcl-2 protein by phosphorylation,[118;119] thereby inducing cytochrome c release from the mitochondrial membrane. Furthermore, ASK1-mediated p38 and JNK activation may phosphorylate Bim and BAX, hence stimulating apoptosis.[120-123] Another connection between the intrinsic and extrinsic apoptosis pathways can be found at the level of caspase-8. Extrinsic signal transduction activates caspase-8, which in turn proteolytically activates the pro-apoptotic member Bid. This Bid then translocates to the mitochondrial membrane, activates the protein Bad, which in turn leads to MOMP and cytochrome c release. Upon formation of the apoptosome, irreversible apoptosis is induced (fig. 4).[107;108;110]

As mentioned earlier, evasion of programmed cell-death is one of the essential alterations that lead to malignant transformation. Besides counterbalancing apoptosis in the onset of a malignancy, this also poses a mayor problem in the treatment of the malignancy. In AML cells, many anti-apoptotic proteins are overexpressed, including Bcl-2, Bcl-XL[124] and NF-κB.[64]

Autophagy

Cell growth and stress responses may, apart from apoptosis, also be regulated through a process called autophagy, which means “to eat oneself”.[125] Autophagy is the major pathway, through which a cell degrades long-lived proteins as well as degrading (damaged) organelles.[126] Nutrient starvation, hypoxia, overcrowding or accumulation of damaged organelles may induce the isolation of parts of the cytoplasm, by wrapping a flat membrane around it, which finally will form a closed structure, called the autophagosome (fig. 5).[125-127] This formation of the pre-autophagosomal structure is class III PI3 Kinase dependent.[127] The autophagosome subsequently fuses with late endosomes, multivesicular bodies or lysosomes, in order to deliver the incorporated cytoplasm to the endo/lysosomal lumen. Lysosomal hydrolases then degrade the contents after which degradation products are transported back to the cytoplasm for recycling.[125-127]

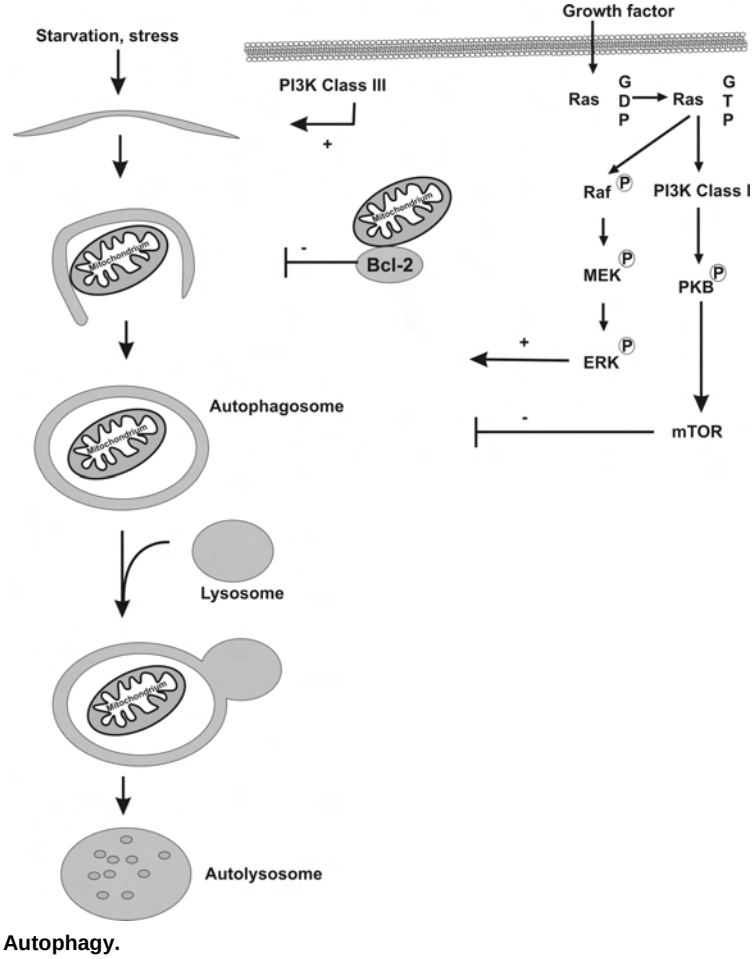

Figure 5 Autophagy.
Schematic representation of autophagy and the signal transduction pathways modulating this process.

Autophagy has long been recognized as a non-apoptotic form of programmed cell death. Whereas apoptotic cell death (type I) is caspase-dependent and characterized by early collapse of cytoskeletal elements, but preservation of organelles until late in the process, autophagocytic cell death (Type II) demonstrates early degradation of organelles with a preservation of cytoskeletal elements.[126] The precise role of autophagy in cancer development is still unclear, but it has been suggested that in early stages of cancer development, autophagy serves a tumour suppressor function. It is hypothesized that in early stages, cancer cells require a higher rate of protein synthesis than protein degradation and therefore autophagy might control tumour growth.[127] Furthermore, autophagy might decrease the mutation rate by

eliminating damaged organelles that induce genotoxic stress through producing free radicals.[127] On the other side, autophagy may promote tumour development in later stages of cancer as a protective mechanism against stressfull conditions.[127] Although autophagy and apoptosis are mostly viewed as separate mechanisms, tumour cells have been observed to undergo both apoptosis and autophagy.[127] Crosstalk may occur as inhibition of apoptosis has been shown to induce autophagy and vice versa.[127] In cancer cells, many of the signalling pathways that regulate proliferation and apoptosis, also regulate autophagy and have been shown to be deregulated. For example, the Ras-Raf-MEK-ERK pathway inhibits apoptosis and stimulates autophagy (fig. 5).[127] On the other side, activated class I PI3 Kinase activates the PKB mTOR pathway and subsequently inhibits autophagy.[125-127] Thus activated Ras signalling may have different effects on autophagy. Furthermore, the anti-apoptotic protein Bcl-2, which is frequently activated in many cancers, has also been shown to be an inhibitor of autophagy, since downregulation of Bcl-2 in the leukemic cell line HL-60 has been shown to enhance autophagy.[128;129]

Although autophagy is predicted to play a major role in cancer, and possibly leukemic development, and targets seem to be available for therapeutic targeting, much about autophagy is still unclear and needs to be clarified. For example, in cancer cells with defective autophagy mechanism, autophagy may be restored in order to induce autophagic cell death, or inhibition of proliferation. Some therapies indeed induce autophagy in leukemic cells,[130] but this may also be an indication of stress survival. In such a case, autophagy needs to be inhibited, demonstrating the importance of clarifying the role of autophagy in cancer.[126;127]

Cell-cycle progression

The cell cycle is composed of 4 phases: the G1, S, G2, and M phase respectively. If the extracellular conditions are favourable and the signals to divide are present, cells will progress through the G1 phase of the cells cycle, pass the restriction point, after which cells are committed to complete the cell cycle and enter the S phase. During S-phase, the genomic DNA is replicated. In the G2-phase the cell prepares to divide. In the M (mitosis)-phase the actual cell division takes place by segregating the two copies of the genome into separate daughter cells followed by cytokinesis where the cytoplasm is divided between the two daughter cells (fig. 6A). The cells enter the G1 phase again and decide to proceed, pause or exit the cell-cycle based on growth promoting or inhibitory signals it receives. When the cell ceases to proliferate, a non-dividing, quiescent state can be entered, known as G0.[131-133]

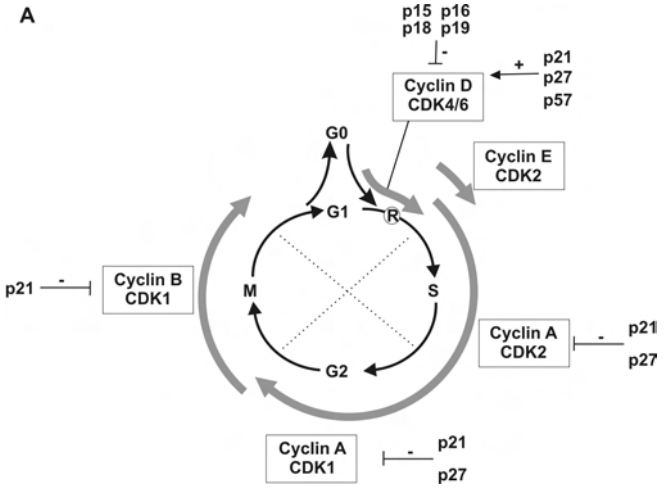

Figure 6A Cell cycle progression.
Schematic representation of cell cycle progression and the involvement of various Cyclins, Cyclin dependent kinases (CDK) and Cyclin dependent kinase inhibitors (CDKi) at various point during the cell cycle. R indicates the Restriction Point. Positive and negative regulation is indicated by + (←) or – (├) signs respectively.

The cell-cycle is controlled by proteins known as cyclins, which associate and activate cyclin-dependent kinases (CDK). Besides their synthesis and destruction at designated points in the cell-cycle, is their activity regulated by cyclin-dependent kinase inhibitors (CDKi).[131] In mammalian cells, 16 cyclins have been discovered and 9 CDKs. The CDKis consist of two families. The first family is known as the Cip/Kip family, made up by p21, p27 and p57. The second family is the INK4 family, consisting of the members p16, p15, p18 and p19, respectively called INK4a to d.[131] Table 4 summarizes interactions between cyclins, CDKs and CDKis as far as their function is correlated to a cell-cycle event.[131;134-136]

Table 4 Cyclins, Cyclin-dependent kinases (CDK) and Cyclin-dependent kinase inhibitors (CDKi).

Cyclin	CDK	CDKi	CDKi regulation	cell-cycle phase	Effect
A	1 and 2	p21, p27	-	S	entry and transition
B1/2	1	p21	-	G2-M	G2 exit, mitosis
C	8			G0-S	transition
D1/2/3	4	p18, p15, p19, p16	-	G0-S	transition
D1/2/3	4	p21, p27, p57	+		Assembly of Cyclin D/CDK complexes
E	2	p21, p27	-	G1-S	transition
F	unknown			G2-M	transition
H	7			All	CDK phosphorylation and activation

When G0 cells receive mitogenic signals from the extracellular environment, for example growth factor-induced Ras-Raf-MEK-ERK signalling, the cell cycle is initiated via upregulation of D-type cyclins.[137-139] Normally, Cyclin D is kept inactive by phosphorylation through GSK-3β, leading to enhanced nuclear export and accelerated degradation through an ubiquitin-dependent proteasomal event.[138] However, Ras activation also induces activation of PI3K-PKB signalling, which effectively inhibits GSK-3β.[132;138] Next, complex formation with CDK 4 and 6 is enhanced by the CDKis p21, p27 and p57 (fig. 6A and B),[131;133;134;140] but in order to being activated and start the transition from G1 into S-phase, the phosphatase CDC25A dephosphorylates the Cyclin D-CDK4/6 complex. [141;142] The primary substrate for the activated Cyclin D-CDK4/6 complex is the tumor suppressor retinoblastoma (Rb).

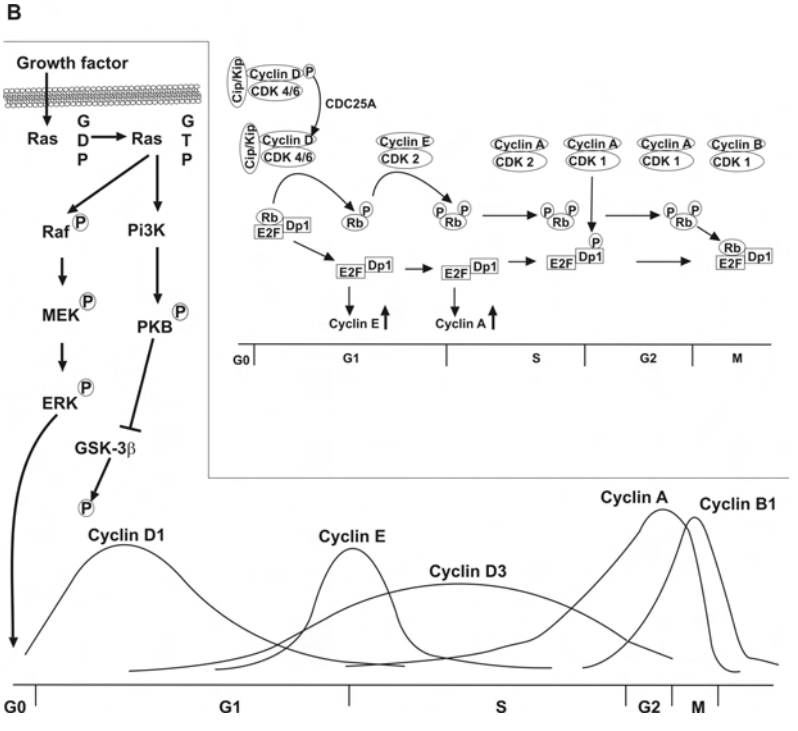

Figure 6B Cell cycle progression.
Schematic representation of complex formation during cell cycle progression and the subsequent modulations on retinoblastoma (Rb) activity. Signal transduction pathways involved in the onset of cell cycle progression are indicated, as well as the level of expression of various cyclins during cell cycle progression.

This Rb protein functions as an inhibitor of the E2F family of transcription factors and prevents the expression of genes involved in cell-cycle progression and DNA synthesis, such as Cyclin E, A, CDK2 and many others.[131] Upon Rb phosphorylation by the Cyclin D-CDK4/6 complex, E2F is released and can start transcription of Cyclin E (fig. 6A and B). Cyclin E subsequently forms a complex with CDK2, which is required to make the final G1-to S-phase transition. This complex hyperphosphorylates the Rb protein, thereby enhancing the positive feedback loop for the accumulation of active E2F.[131] The Rb protein remains hyperphosphorylated during the cell-cycle, until the late M-phase.[143;144] The next target of E2A, Cyclin A is being expressed and associates with CDK2 initially, but later in S-phase with CDK1.[131;133] Cyclin A-dependent kinase activity is not only necessary for the completion of the S-phase and entry into M-phase, but also to phosphorylate the E2F partner DP1,[131;132] which results in shutdown of the transcriptional activity of this complex. The M-phase is regulated by CDK1 in association with Cyclin A, B1 and B2. In order to exit the M-phase, Cyclin A and B are rapidly degraded, upon which the cells can enter the next cycle again (fig. 6A and B) Furthermore, at the end of the M-phase, a phosphatase called PP1 dephosphorylates the hyperphosphorylated Rb protein, so it can bind E2F again.[144;145]

Cell-cycle inhibition

When a cell in G1-phase exits the cell-cycle due to growth inhibitory signals, such as TGF-β or removal of growth factors, the CDKis come into play. Although the Cip/Kip family was above described to assemble the Cyclin D-CDK4/6 complex and hence activate the cell-cycle, they are more famous for their inhibitory actions on the Cyclin E-CDK2 complex. The function for the Cyclin D-CDK4/6 complex is therefore dual: Not only does it initiates the first phosphorylation step of the Rb protein, but it also forms a sink for the Cip/Kip family of CDKis, which therefore are unable to inactivate their target: the Cyclin E-CDK2 complex.[135;139] Upon receiving a growth inhibitory signal, the INK4 family of CDKis associates and inhibits the Cyclin D-CDK4/6 complexes (fig. 6A). A second event directly following from this, is the displacement of Cip/Kip family members from Cyclin D-CDK4/6 to Cyclin E-CDK2 complexes, where it can now start its inhibitory function (Fig 6A).[139] Both Cyclin D and Cyclin E (with their respective CDKs) complexes are now inhibited, which results in an inhibition of phosphorylation of Rb protein. The resulting hypophosphorylated Rb then binds and deactivates E2F again, resulting in cell-cycle arrest in the G0-phase of the cell (fig. 6A and B).[131;132]

Abortion of cell-cycle progression is limited to the early G1-phase. A point of no return was suggested as early as 1974.[146] Nutrient withdrawal before this point, termed the Restriction point, led cells back to the G0-phase of the cycle, but beyond this point cell-cycle progression has become inevitable.[132;133] Although beyond the Restriction point return to G0 is impossible, the Cip/Kip family of CDKis not only inhibits Cyclin E-CDK2 complexes during G1 phase, but also Cyclin A-CDK1/2 and Cyclin B-CDK1 complexes later in the cell-cycle during S and G2-M phase transitions (table 4). This seems to be necessary for G2 checkpoints and prevent reduplication of the genome before mitosis has finished, but will not be further discussed in detail here.[131;136;139;147;148]

Overexpression of cyclins A, D and E, as well as overexpression of CDK4 and 6 have been observed in human cancer and in some forms of leukemia, which may lead to inappropriate Rb inactivation and hence cell-cycle progression.[131;132;149;150] Furthermore, loss of control due to absence of expression of CDKis is frequently observed as well. The INK4 family members p15 and 16 have shown frequent deletions or promoter hypermethylation in ALL and AML, [131;132;135;151] whereas p16 and p14 (p19 in mice) have also shown to be upregulated in AML and ALL, which, when combined, indicates highly aberrant regulation of these proteins.[152-154] The Cip/Kip family members p21 and p27 have been shown to deleted or mislocalized to the cytoplasm in various forms of leukemia as well, which also leads to a loss of cell-cycle control.[131;132;155-157] The above described complex regulation of cell-cycle progression and arrest thus provides various levels at which mutations can occur, leading to uncontrolled growth and hence to a form of cancer.

TGF-β signal transduction

As mentioned earlier, TGF-β can inhibit hematopoietic cell growth. TGF-β directly inhibits the first proliferation divisions of HSC^{LT}, as well as the differentiation of daughter cells.[41;42;158-162] Three TGF-β isoforms exist, TGF-β1, -β2 and -β3, with TGF-β1 being universally expressed. Platelets are an abundant source of TGF-β.[163] Binding of TGF-β to the type II TGF-β receptor, results in recruitment of the type I TGF-β receptor. The type II receptor phosphorylates the type I receptor and activates its kinase activity. This subsequently leads to the activation of Receptor-Smad proteins 2 and 3, which are efficiently recruited to the activated receptor by the SARA (Smad Anchor for Receptor Activation) protein. These R-Smads form a heterodimeric complex with the co-Smad, Smad4, and this complex translocates to the nucleus where it modulates the transcription of many target genes, including genes that affect cell fate (fig. 7).[158;159]

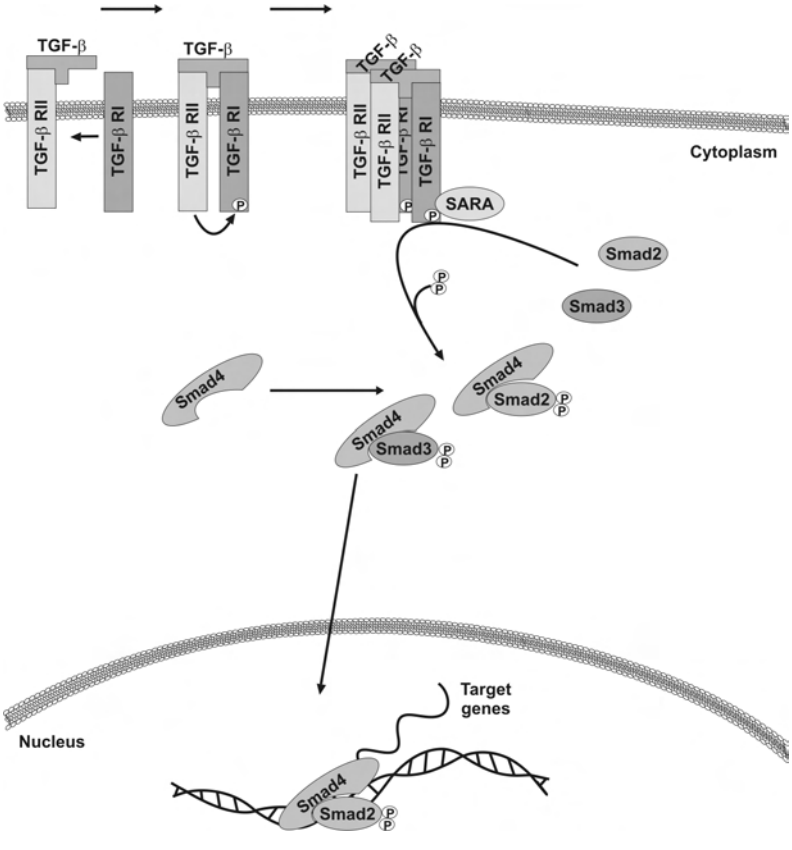

Figure 7 TGF-β signal transduction.
Schematic representation of TGF-β signal transduction. Binding of TGF-β to the TGF-β type II receptor, leads to dimerization with the TGF-β type I receptor. Subsequent tetramerization recruits SARA-Smad complexes to the receptor, which facilitates complex formation of Smad4/Smad2 and Smad4/Smad3 complexes. Subsequent translocation towards the nucleus leads to DNA binding and transactivation of TGF-β target genes.

One of the earliest effects of TGF-β-induced cell cycle arrest is downregulation of c-myc expression by inhibition of transcriptional initiation in early G1, by a complex consisting of Smad3, E2F, the E2F-partner DP1 and the co-repressor p107.[42] Later in G1, expression of various CDKis, including p15Ink4B [164;165], p21Waf1/Cip1 [166;167], p27Kip1 [168;169] and p57Kip2 [170] is upregulated. Furthermore, the expression and activity of additional proteins such as c-Myc, cyclin A, CDK4/6 and the phosphatase cdc25A are suppressed by TGF-β.[171;172] These events eventually lead to decreased activity of the Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes and ultimately result in cell cycle arrest as described previously.

Loss of cell-cycle responsiveness to TGF-β and a possible contribution to the leukemogenic process may occur through inactivating mutations in TGF-β receptors or Smad proteins.[58;158;173] FAB M2 AML subtypes with a t(8;21) translocation (AML-ETO) have been shown to resist TGF-β-mediated transcription, since the AML-ETO fusion protein binds and inactivates Smad3.[174] Furthermore, in FAB M3 subtypes of acute promyelocytic leukemia (APL) it has been demonstrated that the occurring fusion protein PML-RARα can sequester cPML from SARA/Smad2/3 complexes. This cPML is necessary for correct association between SARA, Smad2/3, TGF-β receptor type I and type II interactions and therefore for correct TGF-β signalling.[175] In Chronic myeloid leukemia (CML), the Smad3-transcriptional repressor Evi-1 is upregulated during blast crisis,[176] and the t(3;21) AML-Evi-1 translocation also has been observed to inhibit Smad3-mediated TGF-β-induced growth inhibition.[177] TGF-β-mediated cell cycle arrest can also be blocked by oncogenic Ras. This has been attributed to Map Kinase-dependent phosphorylation of Smad 2/3 and subsequent impaired nuclear translocation, degradation of Smad 4, or mislocalization of p27 to the cytoplasm.[171;178;179]

Although the above described possibilities in abrogation of TGF-β-mediated growth inhibition *in vitro* may contribute to the process of leukemogenesis, in vivo data from the conditional TGF-β receptor type I knock-out suggest that hematopoietic cells are fully functional, despite a growth advantage *in vitro*.[180] This discrepancy between *in vitro* and *in vivo* studies might be explained trough compensational signalling in vivo via ALK-1 (another Type 1 TGF-β Receptor), endoglin (a TGF-β co-receptor) or activins, that have been shown to activate Smad2/3 signalling.[162;180] Therefore, differences between *in vivo* and *in vitro* studies are likely the result from responses of the microenvironment, also since other signal transduction events between HSCs and the bone marrow micro environment, such as Tie2 with Ang-1 respectively, have been shown to render the HSC quiescent.[55]

CDKis and hematopoiesis

In hematopoietic cells other cell biological characteristics, such as apoptosis, differentiation and self-renewal may be affected by regulation of the cell cycle and proliferation by multiple CDKis. The CDKis p21 and p27 for example have been implicated in performing anti-apoptotic functions[181-183] and furthermore has the differentiation of monocytic cells been coupled to the CDKi p21.[183;184]

In the process of self-renewal, p21 has been implicated to maintain the quiescent state of murine HSC, with a subsequent exhaustion of the stem cell pool upon deletion of p21, but this may be dependent upon the mouse

strain.[29;185] The CDKi p18 has been shown to have opposite effects on HSC self-renewal, with a maintenance function upon deletion.[30] Combining both knock-out models indicates that the increased self-renewal after p18 deletion is able to compensate for the exhaustion of stem cells upon deletion of p21.[31] Other INK4 family members, p16 and p19, have shown to be elevated upon BMI-1 deletion,[34] which is a major determinant of self-renewal. The roles of p16 and p19 downstream of BMI-1 are suggested to control proliferation and survival of HSCs during self-renewal divisions, as overexpression of these CDKis have demonstrated a senescent or apoptotic phenotype respectively. Indeed, cells induced to become leukemic loose such an ability upon BMI-1 deletion, due to an elevated proportion of cells in G1 phase of the cell cycle and elevated levels of apoptotic cells.[186] Consistent with this model is the fact that high proliferative clones, which are able to escape this phenotype, have decreased levels of not only p16 and p19, but also decreased levels of p21, p27 and p57.[186]

The CDKi p27 has been implicated in the control of progenitor cell proliferation, instead of HSC pool control as described above, since knock-out mice for p27 have demonstrated an increased progenitor pool.[187] Although HSCs from these mice appear normal in number and cycling activity, upon entering the progenitor stage cycling activity is enhanced with a resulting growth advantage over normal progenitors. Interestingly, more than 9 months after transplantation of p27 deficient cells, a complete dominance was observed in the peripheral blood, whereas the stem cell compartment appeared to be dominated by normal cells.[187] This suggests that the large pool of cells in the periphery is derived from a small pool of cells in the bone marrow. This may resemble an AML, in which a small number of LSCs gives rise to the overwhelming amount of immature progeny. Although p27 deficient mice do not develop leukemia, similar to p21 deficient mice, in concert with other oncogenic hits it may contribute to a leukemia-like phenotype. In AML patients the cytoplasmic to nuclear ratios of p27 have been shown to be a strong prognostic factor for disease free survival (DFS) and overall survival (OS). In patients with high cytoplasmic (and hence low nuclear) p27, both DFS and OS were significantly shorter than in patients with low cytoplasmic to nuclear p27 ratios.[155] Cytoplasmic p27 does not contribute to cell cycle arrest and thus correlates to the observed cycling activity of p27 deficient cells. Although the progenitor phenotype of p27 deficient mice was reproducible, others have also reported an HSC phenotype, especially in combination with deletion of Mad1, an antagonist of the cell-cycle promoting Myc protein.[188]

Trancription factors in hematopoiesis and AML

Nuclear factor-κB

The transcription factor NF-κB has also been implicated to play an important role in cell growth and oncogenesis.[189] In hematopoietic malignancies this is demonstrated by the finding that NF-κB can be constitutively expressed in myeloid leukemic progenitor cells.[64] The dimeric transcription factor NF-κB consists of proteins that are members of the rel family, which comprises five major proteins: p50, p65 (RelA), c-rel, p52 and RelB.[190;191] The most abundant NF-κB dimer is the p50/p65 heterodimer.

There are multiple signals that can activate NF-κB, including cytokines, pathogens, stress and chemotherapeutics.[110;192] As described above, TNFα not only elicits an apoptotic response through the extrinsic pathway, but also anti-apoptotic responses are activated, such as activating NF-κB.[193] In response to for example, TNFα, LPS or IL-1, a variety of cell surface receptors activate the classical NF-κB pathway, through the IKK/IκB/NF-κB signalling pathway (fig. 8).[194;195] Although different receptors often use distinct combinations of intracellular proteins to initiate NF-κB activation, the signals converge into a common pathway that leads to activation of the IκB kinase (IKK) complex.[196-198] This complex consists of a heteromer of related IκB Kinases, IKK1 and IKK2 (Also named IKKα and IKKβ) and the regulatory protein NEMO (also IKKγ).[199-202] This activated complex phosphorylates the inhibitor of NF-κB (IκB) protein, which in an unphosphorylated form, binds to and sequesters NF-κB in the cytoplasm. Upon phosphorylation, IκB is ubiquitinylated and targeted for proteolytic degradation by the proteasome.[203] NF-κB is subsequently released, translocates to the nucleus where it can bind to target DNA sequences in the promoters of target genes (fig. 8).[199;204] Among the target genes of NF-κB are many anti-apoptotic proteins, such as: IAPs (Inhibitor of Apoptotic Proteins; inhibits caspases), Bcl-XL, Bcl-2, cFLIP (FLICE Inhibitory Protein; inhibits caspase-8 activation),[199;205] various growth and differentiation promoting cytokines, such as GM-CSF, G-CSF and EPO[206-208] and various proteins involved in cell-cycle regulation, such as Cyclin D1, D2, D3[209-212] and p21.[183;199;205;213]

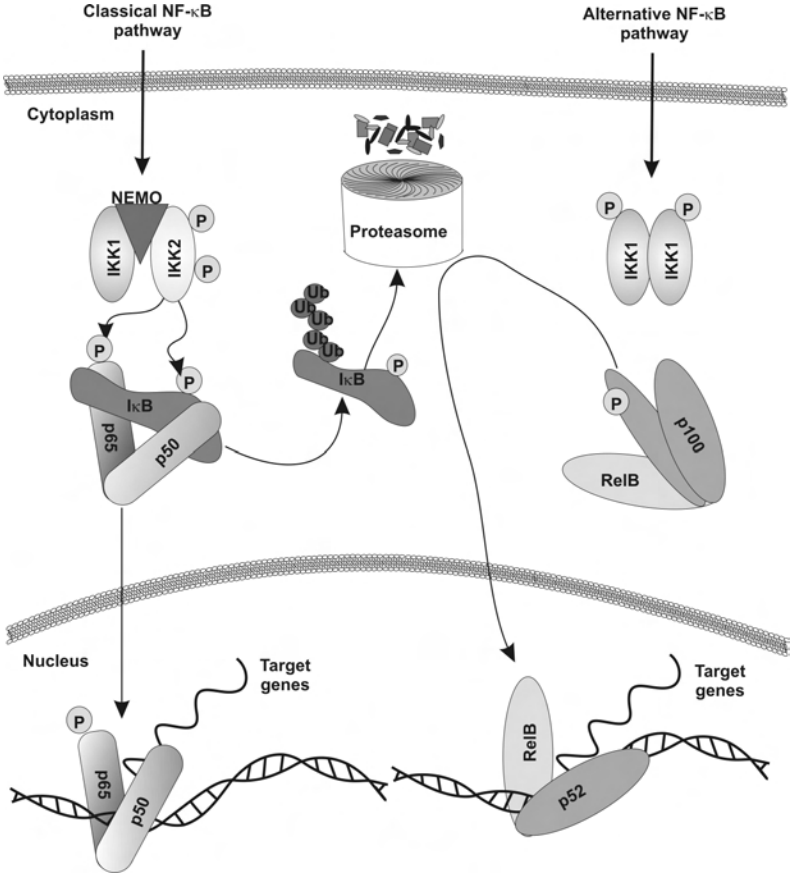

Figure 8 NF-κB signal transduction.
Schematic representation of NF-κB signal transduction via the classical and the alternative pathways. After proteolytic degradation of the inhibitor of NF-κB, IκB, or proteolytic processing of p100 to p52 respectively, subsequent nuclear translocation of activated transcription factor complexes occurs, which results in transactivation of target genes.

A second NF-κB pathway exist which only depends upon IKK1 (fig. 8). A homodimer of IKK1 phosphorylates p100, which is subsequently processed to p52. In combination with RelB, an active transcription complex translocates to the nucleus, where it binds to DNA-target sequences.[204;214] In AML aberrant regulation of the classical NF-κB pathway through IKK2 signalling has been shown to lead to increased NF-κB activity,[215] which has been observed in the majority of the AML cases and resulting in resistance to apoptosis inducing signals, such as chemotherapy.[64;216]

Signal Transducer and Activator of Transcription 5

A transcription factor involved in many of the above described processes in hematopoiesis is STAT5, a member of the Signal Transducer and Activator of Transcription (STAT) family. This family contains 7 members, STAT 1 to 6, with STAT5 comprising STAT5A and STAT5B.[217] Induction of STAT5 transactivation has been demonstrated to occur after stimulation with multiple growth factors and cytokines, including FLT3-L, SCF, G-CSF, GM-CSF, IL-3, IL-6, EPO and TPO and affects differentiation, self-renewal, apoptosis and proliferation of human hematopoietic stem/progenitor cells.[218-224] These differences in outcome after STAT5 activation find their origin in differences in cell type, (combinations of) cytokine signals and concentrations of cytokines.[225;226] Upon binding of growth factors or cytokines to their receptors, dimerization of the receptor takes place. Since cytokine receptors and many growth factor receptors lack intrinsic kinase activity, Janus Kinases (JAKs) are recruited to the receptors. Cross-phosphorylation in turn activates the tyrosine kinase activity of these JAKs, which then phosphorylate tyrosine residues in the cytoplasmic tail of the receptor. These phosphorylated tyrosine residues act as docking sites for STAT molecules, which subsequently become phosphorylated by JAKs as well. These phosphorylated STAT5 monomers dimerize, translocate to the nucleus, bind to specific sequences in the promoter of target genes and activate transcription (fig. 9).[217;225-227] Among the STAT5 targets are genes for cell-cycle regulation: Cyclin D1, D2, D3, p21;[228-232] anti-apoptotic genes: Bcl-2, Bcl-XL and XIAP;[37;233] genes involved in (erythroid) differentiation such as hemoglobin genes[37] and many more. Although the upregulation of p21 seems odd to progress cell-cycle, STAT5 has been shown to induce low expression of p21, necessary for assembly of Cylin D-CDK4/6 complexes and the promotion of cell-cycle[134;232]

Since constitutive STAT5 activation has been observed in many AML patients,[40;234] which may be due to activating mutations in upstream kinases[235-237] or alternatively result from autocrine growth factor production,[40] it is interesting to further investigate the role of STAT5 in AML. From the presence of normal peripheral blood counts in STAT5AB knock-out mice it may be concluded that STAT5 is either not necessary or redundant for hematopoiesis, even though cytokine responsiveness was abrogated in STAT5AB knockout cells[238] and colony assays demonstrated reduced numbers of cytokine-responsive myeloid progenitors.[239] In competitive repopulation experiments however, these cells demonstrated a severe impairment in engraftment, [240;241] suggestive for a role of STAT5 in self-renewal. This was demonstrated when constitutively activated STAT51*6 caused a self-renewal phenotype in human

and mouse HSCs, which developed into a myeloproliferative disease (MPD) in murine transplantation models.[36;37] Besides effects on self-renewal and proliferation in hematological diseases,[37] STAT5 activation may contribute to anti-apoptosis, since in a model for Polycythemia Vera (PV), it was recently shown that downregulation of either STAT5 or its target gene Bcl-XL was sufficient to inhibit erythroid colony formation in the absence of EPO. This was demonstrated to occur upon mutation of JAK2, one of the upstream kinases of STAT5.[242;243] Interestingly, the overexpression studies of constitutively activated STAT51*6 have shown a predominant erythroid differentiation program, which is consistent with EPO-independent growth of PV.[37]

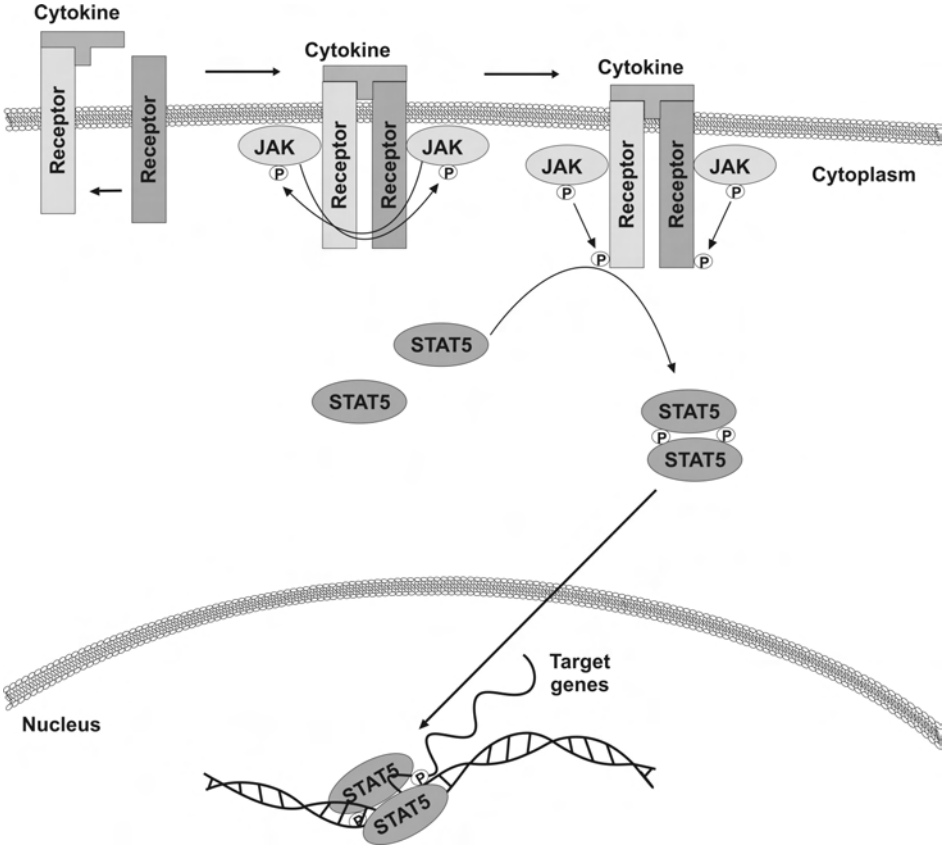

Figure 9 STAT5 signal transduction.
Schematic representation of STAT5 signal transduction. Cytokine-induced receptor dimerization leads to activation of JAK kinases. Phosphorylation of the receptor forms a docking site for STAT5 monomers, which can subsequently be phosphorylated. Phosphorylated STAT5 hence forms dimers and translocates to the nucleus, where the complex can bind DNA and transactivate transcription of target genes.

CCAAT enhancer binding protein (C/EBP) α

Besides the above described transcription of target genes and subsequent changes in apoptosis and proliferation, it was recently shown that STAT5 activation can also reduce the expression of the transcription factor C/EBPα.[37;244] C/EBPα is leucine zipper containing transcription factor, via which it can homo- or heterodimerize[245] and is decisive for myeloid differentiation in the hematopoietic system, through inducing differentiation, arresting the cell-cycle and enhance cell-survival.[246-249] C/EBPα expression is upregulated when CMPs and GMPs develop.[8;246] Overexpression in cells with GMP potential indicated that C/EBPα can induce granulocytic differentiation and subsequently block the monocytic differentiation program,[246] although recently overexpression studies indicated that C/EBPα could direct monocytic development as well.[247] The reduced C/EBPα expression hence correlates with the block in myeloid differentiation and enhanced erythroid differentiation observed in STAT51*6 overexpressing cells. The C/EBPα reduction is not only necessary for reduction of expression of myeloid genes, normally expressed through C/EBP, but it was demonstrated that STAT5 and C/EBPα have opposite effects on cell-cycle as well.[244] C/EBPα cooperates with p21 to induce a cell-cycle arrest[250] and high levels of p16 have been correlated to high levels of C/EBPα as well.[251] Furthermore has C/EBPα been shown to interact with E2F, CDK2 and CDK4 to slow G1 progression.[252]

Furthermore, like STAT5, C/EBPα has been implicated in the regulation of HSC self-renewal, since the repopulating ability of HSCs lacking this transcription factor was increased as compared to wildtype (wt) HSCs.[253] Also, these mice demonstrated an accumulation of immature myeloid blasts in the bone marrow upon transplantation with C/EBPα$^{-/-}$ HSCs,[253] which is consistent with an AML-like phenotype. Interestingly, C/EBPα is negatively regulated in various AML subtypes. Mutations in the N-terminus have been described to occur in patients with a FAB M1 subtype as well as in FAB M2 without AML-ETO translocation.[81;254;255] These N-terminal mutations give rise to a truncated dominant negative form of C/EBPα , which interferes with DNA-binding and transactivation of the wt C/EBPα.[254] C-terminal mutations of C/EBPα have been described in FAB subtypes M1, M2 and occasionally in M4 and result in deficiency in DNA binding of C/EBPα. [256;257] AMLs with an AML-ETO translocation have been demonstrated to downregulate C/EBPα at the transcriptional level,[81] although some studies have also shown that AML-ETO can associate with and inhibit C/EBPα function.[258] In AML patients with Flt3-ITD mutations, suppression of C/EBPα expression was demonstrated,[259] whereas functional inhibition of C/EBPα through Flt3-ITD-induced

phosphorylation has also been reported.[260] Lastly, the fusion gene AML1-MDS1-EVI1, observed in patients with AML, MDS or CML, has been demonstrated to suppress DNA binding of C/EBPα (fig. 10).[261] The interference with C/EBPα function in myeloid differentiation of many AMLs, combined with constitutive activation of STAT5, indicates the importance of an appropriate balance between these two transcription factors in normal hematopoietic stem cells to prevent deregulation towards a leukemic phenotype.

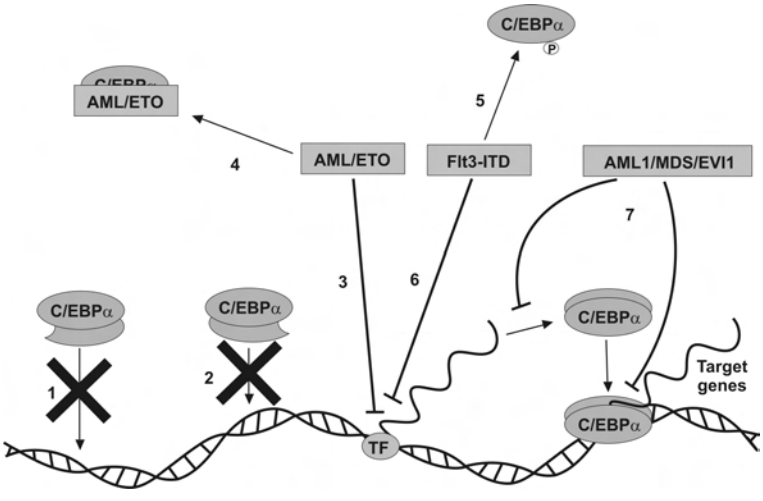

Figure 10 Mechanisms of C/EBPα inhibition.
Schematic representation of various mechanism present in leukemic cells to impair transcription of C/EBPα target genes. Shown are: 1) N-terminal dominant negative mutations of C/EBPα, 2) C-terminal dominant negative mutations, 3) AML-ETO-mediated transcriptional downregulation of C/EBPα mRNA, 4) AML-ETO binding to C/EBPα and subsequent interference with DNA binding, 5) Flt3-ITD-mediated phosphorylation, 6) suppression of C/EBPα expression in Flt3-ITD positive AMLs by an unknown mechanism and 7) inhibition of C/EBPα translation as well as the transcription function of C/EBPα by AML1/MDS/EVI1 translocation products.

Scope of this thesis

As is evident from the above described pathways, normal hematopoiesis as well as Acute Myeloid Leukemic hematopoiesis is a complex integration of various signals, both resulting from extrinsic influences, e.g. growth factor signalling or interactions with the microenvironment and intrinsic cues, such as epigenetic modifications and many more. During the last years, considerable progress in the understanding of protein expression, localization, activation and their involvement in processes such as proliferation, apoptosis, differentiation and self-renewal has been made. Although more and more data are available and many more proteins have been assigned a role in specific signal transduction pathways, the overall picture has also become more complex. Signal transduction pathway activation has gained a cell-type and localization specific dimension. And previously unexpected roles of proteins have now been assigned in other processes then those widely assumed. For example: anti-apoptotic roles of cyclin-dependent kinase inhibitors when localized in the cytoplasm of specific cell types is significantly different from the assigned growth inhibitory actions when situated in the nuclear compartment of almost all cell-types.

To understand the molecular mechanisms that are involved in the process of leukemic transformation, as well as in understanding how these derailments from normal hematopoiesis can potentially be counteracted by clinical treatment, detailed analysis and intervention studies are of considerable importance. However, AML is a complex and multi-facetted disease, which makes it difficult to study. Studies that focus solely on one process such as apoptosis, proliferation, differentiation or self-renewal might highlight some aspects that underlie the development of leukemia, but will most certainly underestimate how the interplay of multiple molecular signal transduction pathways ultimately results in the disease. This thesis involves studies regarding apoptosis, proliferation, differentiation and self-renewal, with an attempt to reconcile observations in primary AML blasts with detailed molecular mechanisms from other studies. Since AML blasts have shown resistance to apoptotic stimuli and the transcription factor NF-κB is frequently found to be activated, in **chapter two** we investigated the signal transduction pathway involved in activation of this transcription factor.

Since this transcription factor is so frequently activated in AML and a multiplicity of constitutively activated signal transduction pathways converges on NF-κB, **chapter three** investigates what the effects of constutive activation of NF-κB are on normal hematopoiesis. Is NF-κB activation sufficient as a

single hit to impair normal hematopoiesis, as has been suggested by inactivation studies in normal hematopoietic cells? Overexpression studies of constitutive active mutants are used to investigate apoptosis, proliferation, differentiation and self-renewal of cord blood-derived CD34+ stem and progenitor cells.

The cyclin-dependent kinase inhibitor p21 has been shown to direct monocytic differentiation, due to an anti-apoptotic function when cytoplasmically localized. This prompted us to investigate whether AMLs from the monocytic subclassifications demonstrated aberrant cytoplasmic localization of p21 and whether they were less susceptible to apoptotic insults as a result from cytoplasmic localization of p21. This has been described in **chapter four**.

Aberrant expression of Heat Shock Proteins has been observed in AML as well as in other malignancies and the last few years coupled to the inhibition of apoptosis via various pathways. We wondered whether HSP27 was differently expressed in AML and if it contributed to the insensitivity towards chemotherapeutics. In **chapter five** we describe the use of RNA interference to study the apoptotic pathways inhibited by HSP27.

As shown in chapter 2, Ras signal transduction is frequently activated in AML blasts. Ras is activated in response to many growth factors and has a cell-cycle promoting effect. Because multiple studies in hematopoietic cells have indicated the importance of TGF-β as an inducer of cell-cycle arrest and AML cells are unresponsive towards TGF-β, **chapter six** investigates via which intermediates Ras signal transduction inhibits the response towards TGF-β.

AML is thought to emerge from hematopoietic stem cells with, amongst others, aberrant self-renewal and limited differentiation capacities. Recently, the transcription factor STAT5 was shown to be involved in self-renewal and to induce a block in myeloid differentiation. Since STAT5 is activated in approximately 66% of the AML cases, we investigated what the effects of down regulation of STAT5 protein levels would be on normal and leukemic stem cells. This has been described in **chapter 7**.

Furthermore, STAT5 activation resulted in the downregulation of C/EBPα, a transcription factor that is involved in myeloid differentiation. This C/EBPα has been shown to be deregulated in almost all AML subtypes and therefore it would be interesting to investigate what would happen if C/EBPα levels were to be restored in leukemic stem cells. The effects on differentiation, growth and self-renewal were examined and described in **chapter 8**.

In the last chapter, **chapter 9**, the data are summarized and put in perspective of the field of hematology.

Constitutive NF-κB DNA binding activity in AML is frequently mediated by a Ras/PI3-K/PKB-dependent pathway

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Chapter 2

Abstract

In the present study we aimed to elucidate the mechanism responsible for constitutive NF-κB DNA binding activity in AML cells. Intervening in aberrant signalling pathways provides a rational approach for in vivo targeting of AML cells. Constitutive NF-κB DNA binding activity was observed in 16 of 22 (73%) investigated AML cases and was in general associated with resistance to spontaneous apoptosis. Indeed, inhibition of NF-κB activity by the NF-κB inhibitor SN-50 peptide resulted in enhanced chemotherapy-induced apoptosis. In the majority of the cases constitutive NF-κB activity was mediated by a Ras/PI(3) Kinase/Protein Kinase B (PKB)-mediated pathway. The PI3-K inhibitor Ly294002 and the Ras inhibitor L-744832 both inhibited PKB phosphorylation and NF-κB DNA binding activity. The constitutive activation of Ras GTP-ase was in 29% of the cases caused by mutations in the gene encoding for N-Ras. The constitutive NF-κB activity could so far not be ascribed to the autocrine production of growth factors or to mutations in the Flt3 receptor since anti-GM-CSF,-IL-1, -IL6, -TNFα or the tyrosine kinase inhibitor AG1296 did not affect the NF-κB DNA binding activity.
The present study demonstrates that Ras activation is an important pathway for triggering the NF-κB pathway in AML cells.

Introduction

The transcription factor nuclear factor kappa B (NF-κB) has been studied intensively for its role in controlling expression of genes involved in immune and inflammatory function.[192;194;262] Recently, NF-κB has also been implicated to play an important role in cell growth and oncogenesis.[189] In hematopoietic malignancies this is demonstrated by the finding that NF-κB can be constitutively expressed in myeloid leukemic progenitor cells.[64] NF-κB is a dimeric transcription factor that consists of proteins that are of members of the rel family, which comprises five major proteins: p50, p65 (RelA), c-rel, p52 and RelB.[190;191] The most abundant NF-κB dimer is the p50/p65 heterodimer.

There are multiple signalling pathways that can potentially activate NF-κB. First, the IKK/IκB/NF-κB signalling pathway can be activated through stimulation of a variety of cell surface receptors.[194;195] Although different receptors often use distinct combinations of intracellular proteins to initiate NF-κB activation, the signals converge into a common pathway that leads to activation of the IκB kinase (IKK) complex.[196-198] In acute myeloid leukemia (AML) aberrant regulation of IKK signaling has been shown to lead to increased NF-kappaB activity.[215] In addition to the classical NIK/IKK/IκB pathway, induced by IL-1 or TNFα, it has been described that downstream effectors of the small Ras-GTP-ase can also interfere with the NF-κB pathway.[263-265]

Activating point mutations of the Ras genes have been demonstrated in 20%- 30% of acute myeloid leukemia cases, which are mostly mutations in the N-Ras gene, but occasionally mutations in K-Ras have been described.[266;267] Ras activation of the Raf serine/threonine kinases and the activation of the ERK mitogen-activated protein kinases (MAPKs) remains a key signalling pathway that is important for many aspects of Ras effector functioning.[268] An additional signal transduction effector of Ras is the PI3-K/PKB signalling pathway.[269] PI3-K can also activate the Rac GTP-ase, and this Rho family protein is an important mediator of oncogenic Ras transformation.[270] PKB, but also Rac, facilitates Ras activation of NF-κB in some cellular settings, and serves an anti-apoptotic role in Ras function.[270 271] This is in line with observations which describe that in the AML-derived HL60 cell line the PI3-K/PKB pathway plays an important role in chemoresistance, which is distinct from interactions between the pro-apoptotic protein Bad with Bcl-2 family members. Thus the PI3-K/PKB pathway is suggested to have different targets.[272]

It has previously been reported that NF-κB is constitutively activated in leukemic progenitor cells. More importantly, the activation of NF-κB is a major distinguishing characteristic between normal and leukemic stem cells and provides leukemic cells a growth advantage over the normal hematopoietic stem cell. Therefore, it is important to clearly identify the role and especially the regulation of NF-κB in AML. The identification of these signaling cascades might lead to a more rational approach to interfere with the aberrant proliferation and cell survival characteristics of leukemic cells. The results of the present study demonstrate that constitutive PI(3)K dependent activation of NF-κB DNA binding activity in AML is triggered by Ras activation in the majority of cases. Activating mutations in the Ras gene contribute only in the minority of the cases.

Materials and methods

Patient population and isolation of AML cells - Peripheral blood cells or bone marrow cells from 22 adult, untreated, patients with AML were studied after informed consent. The AML cases were defined according to the classification of the French-American-British (FAB) committee as M0-M6.[18] AML blasts were isolated by density-gradient centrifugation. The cells were cryopreserved in aliquots of 20-30 x 10^6 cells in RPMI 1640 (Flow, Rockville, MD) supplemented with 10% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), employing a method of controlled freezing and storage in liquid nitrogen. After thawing, T lymphocytes were depleted by 2-aminoethylisothioronium bromide (AET)-treated sheep red blood cell (SRBC) rosetting. The cell population consisted of more than 98% AML blasts as determined by May-Grünwald-Giemsa staining. Fluorescence-activated cell sorting (FACS) analysis demonstrated <1% CD3 (Becton Dickson, Sunnyvale, California, USA) positive cells. AML blasts were cultured at 37°C at a density of 1x10^6/ml in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (ICN, Costa Mesa, CA, USA), 6 ng/ml of colistine and 10% FBS.

Cell culture - The human erythroleukemia cell line TF-1 was cultured in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (ICN, Costa Mesa, CA, USA), 6 ng/ml of colistine, 5% FBS and 10 ng/ml IL-3 (Genetics Institute Cambridge, MA). The human monocytic cell line THP-1 was

cultured in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (ICN, Costa Mesa, CA, USA), 6 ng/ml of colistine and 10% FBS.

Combined annexin V/PI staining procedure - Viability was assessed by using an annexin V staining kit (IQ Products, Groningen, The Netherlands) according to the manufacturer's recommendations. Briefly, after 24 hours of culture in RPMI 1640 medium supplemented with 10% FBS with or without addition of VP-16 (50µg/ml) (TEVA Pharma) or the specific NF-κB inhibitor SN-50 (100µg/ml) (Biomol, Plymouth Meeting, PA, USA), cells were harvested, resuspended in 100 µl calcium buffer containing 5 µl of annexin V and incubated for 20 min at 4°C in the dark. Cells were washed with 5 ml calcium buffer and subsequently incubated in 300 µl calcium buffer containing 2.5 µl of propidium iodide (PI) for 10 min in the dark. Subsequently, binding of fluorescein-conjugated annexin V and PI was measured by fluorescence-activated cell sorting (Becton Dickinson, Sunnyvale, CA). The specificity of the SN-50 effect was tested by stimulating monocytes with the phorbol ester PMA (50 nM, Sigma, St. Louis, MO, USA) during 6 hours that were pre-incubated with or without SN-50. Subsequently the NF-κB activity was studied by EMSA and p21 (Transduction Laboratories, Lexington, KY, USA) by western blotting.

Western blotting - The amount of ERK, PKB, p21, actin and the degree of phosphorylated ERK, PKB and STAT5 were determined by western blotting. AML blasts were cultured for 24 hours in RPMI 1640 supplemented with 10% FBS, with or without 50µM L-744832 (Biomol, Plymouth Meeting, PA, USA), 5µM Ly294002 (Alexis Biochemicals, Switzerland), 20µM AG1296 (Calbiochem,La Jolla, CA, USA) or 10µM PD98059 (Calbiochem,La Jolla, CA, USA) at a density of $1x10^6$ cells/ml. TF-1 cells were cultured overnight in RPMI 1640 supplemented with 1% FBS and subsequently stimulated for 5 minutes with IL-1β (10 ng/ml) or conditioned AML medium (prepared by incubating AML cells at a density of $3x10^6$ cells/ml for 3 days). $3x10^6$ cells were harvested and total cell extracts were prepared by resuspending the cells in 100 µl 1x Sample buffer (containing 2% SDS, 10% glycerol, 2% ß-mercapthoethanol, 60mM Tris-HCl pH6.8 and bromophenol blue). Cell extracts were directly boiled for 5 minutes and proteins were fractionated by SDS PAGE. The proteins were electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA) and probed with antibodies against phospho Thr308-PKB, phospho Ser473-PKB, phospho Ser32-IκB-α, phospho Thr202/Tyr204-ERK (E10) (which recognizes both residues on both ERK1 and 2)(New England BioLabs, Beverly, MA, USA)or phospho Tyr694/Tyr699-STAT5 (Upstate, Charlottesville, VA, USA)

(which recognizes the phosphorylated tyrosine 694 residue of STAT5A and the phosphorylated tyrosine 699 residue of STAT5B), PKB (New England Biolabs, Beverly, MA, USA), IκB-α (C-15) or ERK (K23) (Santa Cruz, CA, USA), STAT5A, STAT5B (Upstate, Charlottesville, VA, USA) or monoclonal antibody against Actin (C4) (ICN Biomedicals, Inc., Aurora, Ohio, USA). Immunocomplexes were detected using enhanced chemiluminescence (ECL, Amersham). Relative protein levels were quantified using the gelscan program Diversity One (Pharmacia,Uppsala, Sweden).

Electrophoretic mobility shift assay - AML blasts were cultured for 24 hours in RPMI 1640 supplemented with 10% FBS, with or without addition of Ly294002 (5μM), L-744832 (50μM), PD98059 (10μM), SB203580 (1μM), AG1296 (20μM), anti-GM-CSF antibody (1μg/ml) (R&D Systems, UK), anti-IL-6 (1:500) (gift from Dr. L. Aarden, CLB, Amsterdam), anti-IL-1β (0.1μg/ml), anti-TNFα (1:100). The diluted polyclonal antisera were maximally effective at the used concentrations as studied with in vitro dose-response curves. TF-1 cells were cultured overnight in RPMI 1640 supplemented with 1% FBS and subsequently stimulated for 15 minutes with conditioned AML medium (prepared by incubating AML cells at a density of $3x10^6$ cells/ml for 3 days) or IL-1β (10 ng/ml). THP-1 cells were cultured overnight in RPMI 1640 supplemented with 1% FBS and subsequently stimulated with Flt3 ligand (100ng/ml) (a friendly gift from Immunex Corporation, Seattle, WA). Nuclear extracts were prepared according to the mini-scale procedure described.[273] Nuclear extracts were divided into small aliquots and stored at -80°C. Cytoplasmic extracts were normalized for protein content and analyzed for the amount of phosphorylated IκB by western blotting.

Double-stranded synthetic oligonucleotide probes containing the NF-κB (NF-κB: 5'-AGCTGCGGGGATTTTCCCTG-3') consensus sequence (underlined) were used in the gel retardation assay. Fifty ng of HPLC-purified single-stranded oligonucleotide were labeled with T4-polynucleotide kinase and [α^{32}p]dATP (3000 Ci/mmol, Amersham), separated from non-incorporated radiolabel by sephadex G50 chromatography, ethanol precipitated, dried, and dissolved in 20 μl of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, containing a four fold excess of the opposite strand. Annealing of the two complementary strands was performed by heating the mixture for 2 minutes at 90°C and slow cooling to room temperature. Five μg of nuclear extract and 0.1 ng double-stranded labeled oligonucleotide were incubated in 20 mM HEPES (pH 7.9), 60 mM KCl, 0.06 mM EDTA, 0.6 mM DTT, 2 mM spermidine, 10 % glycerol, supplemented with 2 μg poly(dI-dC).

The binding reaction was performed at 26°C for 25 minutes. The samples were loaded on pre-run (30 min, 100 V) 4% (30:1) polyacrylamide gels and run for 1 hour at 150 V in 0.5 x TBE at room temperature. Gels were dried and exposed to Kodak XAR films at -80°C with an intensifying screen. Quantification of protein binding was performed by densitometry using a Phospho Imager (Molecular Dynamics, Sunnyvale, CA). Cold competition (cc) experiments were performed by adding a 100 fold molar excess of unlabeled self double-stranded oligonucleotides. Supershift experiments were performed by incubating the nuclear extracts with polyclonal antibodies against p50 and p65 subunits of NF-κB (Santa Cruz).

Ras binding reaction - AML cells ($5x10^6$) and cell lines were incubated in 2 ml RPMI 1640 medium supplemented with 0.5% FBS for 24 hours. Subsequently, cells were harvested and lysed in 400 µL lysis buffer (10% glycerol, 1% NP-40, 50mM Tris/HCl pH 7.5, 200mM NaCl, 2mM MgCl2, 2µg/mL aprotinin and protease inhibitors) for 10 minutes on ice. Cell lysates were clarified for 10 minutes at 4°C by centrifugation at 10,000g and incubated for 1 hour with 10 µg of Raf-GDS glutathione-S-transferase-Ras binding domain (GST-RBD) precoupled to glutathione-sepharose beads (Pharmacia). Subsequently, the beads were washed once with lysis buffer and 20µL of SDS-sample buffer was added to the beads. The bound proteins were resolved on 15% SDS-PAGE gel and detected by immunoblotting using N-Ras antibody (Santa Cruz).
Bacterial expression and purification of the Raf-GDS GST-RBD fusion protein (a friendly gift from P. Coffer, Utrecht, The Netherlands) has previously been described.[274]

Mutation analysis of N- and K-Ras - Mutation analysis of N-Ras was performed by single-strand conformation polymorphism (SSCP). Genomic DNA was extracted from AML cells as previously described.[40] PCR analysis was performed using primers 1FW 5'-CAGGTTCTTGCTGGTGTG-3' AND 1BW 5'-GCTACCACTGGGCCTCAC-3' for amplification of a 149-bp exon 1 fragment containing codons 12 and 13.
The primers 2FW 5'-CAAGTGGTTATAGATGGTG-3' and 2BW 5'-ATAATGACTCCTAGTACCTG-3' were used to amplify a 181bp exon 2 fragment containing codon 61. The PCR reaction was performed using 200 ng of genomic DNA in a total volume of 25 µl using 0.5U of Taq polymerase (Gibco BRL, Grand Island, NY) and 0.1µl label (^{32}Pd-CTP) according to manufacturers recommendations. For SSCP analysis 5 µl PCR product was denatured for 2 minutes at 96°C with an equal volume stop solution (95%

Formamide, 10 mM NaOH, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol) and then placed on ice. A volume of 5µl sample was loaded onto a 0.5xMDE polyacrylamide gel (FMC Bioproducts) and electrophoresed at room temperature for 16 hours at 250V. After electrophoresis a Kodak film was exposed to the gel for 16 to 24 hours. Samples that demonstrated shifted single stranded DNA fragments were amplified again without radiolabeling, and purified using a PCR purification kit (Qiagen Inc, Chatsworth, CA). Subsequently, DNA fragments were sequenced in both directions using an ABI 377 automated sequencer.
Mutation analysis of the complete coding region of the K-Ras gene was performed according to a protocol using denaturing gradient gel electrophoresis (DGGE) analysis as described before.[275]

Immunoprecipitation - $2x10^7$ AML blasts were cultured for 24 hours in RPMI 1640 medium supplemented with 10% FBS. Cells were harvested, washed with ice-cold PBS containing 1 mM sodium orthovanadate and subsequently lysed in 500µl lysis buffer (50 mM HEPES, pH 7.4, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 25 mM NaF, proteinase inhibitors (Complete: Boehringer Mannheim, Germany), 1µM pepstatin, 1mM sodium orthovanadate) for 10 min on ice. Cell lysates were clarified at 20 000g for 20 min and then precleared with 30 µl Protein A Sepharose beads (50% slurry) for 1 hour at 4°C. After 5 min centrifugation at 380 rpm, the cell lysates were incubated with 5 µL Flt3 antibody (C-20: Santa Cruz, CA, USA) rotating overnight at 4°C. Protein A sepharose beads (30µl) were added to each sample and incubated for another 4 hours at 4°C. The immune complex was washed 3 times with lysis buffer. 1/10th of the precipitate was analyzed for the amount of precipitated Flt3 protein and 9/10th of the precipitate was analyzed for the degree of tyrosine phosphorylated Flt3. The immune complexes were heated in sample buffer, separated by SDS PAGE, immunoblotted on PVDF membrane (Millipore, Bedford, MA) and incubated overnight with either anti-phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY) or anti-Flt3 antibody. Immunocomplexes were detected using ECL (Amersham, UK).

Analysis of the internal tandem duplication of the Flt3 gene - Genomic DNA was extracted from AML cells and Flt3 gene duplications were identified by allele specific PCR amplification followed by PAGE analysis.[40] Therefore, PCR amplification was performed by using the forward primer for exon 11: 5'-GCAATTTAGGTATGAAAGCCAGC-3' and the reverse primer for exon 12: 5'-CTTTCAGCATTTTGACGGCAACC-3' comprising the complete exon 11-12 region of the Flt3 gene which contain internal tandem duplications as described

by previous studies.[40;235;276] The PCR was performed using 200ng of genomic DNA in a total volume of 50 µl using 1U of Taq polymerase (Gibco BRL, Grand Island, NY) according to manufacturers recommendations.

The amplified PCR product was run on a 7.5% acryl/bisacrylamide gel (29:1) cut out from the gel and dissolved in 40 µl H_20 overnight at 4°C. 20µl of this dissolved PCR product was used for another PCR reaction under the same conditions as described above. The amplified PCR product was purified with a PCR purification kit (Qiagen Inc, Chatsworth, CA) and directly sequenced using the forward primer for exon 11.

Statistical analysis - The student t-test was used to determine the significance of the apoptosis data in conjuction with NF-κB positive and negative samples. To determine if levels of phosphorylated PKB after L-744832 and Ly294002 treatment differed from control cells, densitometric analysis was followed by a student t-test. To demonstrate a correlation between the expression patterns of different proteins the Spearman's Rank correlation coefficient was determined.

Table I FAB classifications, percentage of apoptotic cells, phosphorylation and activation patterns and Ras and Flt3 gene mutations of patients with AML.

AML case	FAB	Apoptosis %	NFκB	Ras activity	Ras mutation	ERK phos.	PKB phos.	Flt3 mutation	Flt3
1	M2	14	+++	+/-	-	-	+++	-	+
2	M4eo	23	++	+	-	+++	+++	-	-
3	M4	24	+	+	-	+++	+++	+	+
4	M6	26	+	+	-	-	-	+	+
5	M1	20	+	++	-	+	-	+	+
6	M5	21	+++	+	+ (N12)	++	+++	-	-
7	M4	30	+	++	-	+	+/-	+	+
8	M2	9	+	++	-	+/-	+	-	nd
9	M2	24	+	+/-	-	++++	++	-	-
10	M1	30	++	+++	+ (N13)	+++	+	-	+
11	M4	13	+/-	+	+ (N61)	+++	+/-	-	-
12	M4	50	++	+++	-	+++	+	+	-
13	M2	40	+	++	+ (N12)	++	++	-	-
14	M4	38	+	-	-	+++	+++	-	+
15	M2	59	+	-	-	+	-	+	+
16	M2	45	+++	-	-	-	-	-	+
17	M1	61	-	-	-	-	-	-	-
18	M2	39	-	+	-	-	+/-	+	+
19	M1	67	-	+/-	-	+	-	+	+
20	M4	69	-	-	-	+	++	-	-
21	M1	33	-	-	-	++	-	+	+
22	M2	57	-	-	-	+	-	+	-
TF-1		20	+	++	+ (N61)	++	+/-	-	nd
THP-1		25	+/-	++	-	+/-	+/-	-	nd

FAB = French-American-British classification, NF-κB = analysis of NF-κB DNA binding activity as determined by EMSA, described in material and methods; Ras activity is determined by the Ras binding reaction described in material and methods; Ras mut. = Mutation of Ras as is determined by the analysis described in material and methods, (12), (13), (61) = N-Ras mutation in codon 12, 13 or 61; ERK phos. = analysis of phosphorylated Thr202/Tyr204-ERK; PKB phos. = analysis of phosphorylated Ser473-PKB; Flt3 mut. = analysis of internal tandem duplications (ITD) as described in material and methods; Flt3 = analysis of Flt3 receptor activation as described in material and methods, nd = not done.

Results

Constitutive NF-κB DNA binding activity in AML blasts

Blasts of 22 patients with AML (FAB classifications: M1 (n=5), M2 (n=8), M4 (n=7), M5 (n=1), M6 (n=1)) and two cell lines (TF-1, THP-1) were studied with regard to their constitutive NF-κB DNA binding activity by means of electrophoretic mobility shift assays (EMSA). The specificity of the NF-κB DNA binding was shown by competition assay by adding a 100 fold molar excess of unlabeled oligonucleotide (fig. 1B). In addition no difference was observed for NF-κB expression in fresh AML samples versus in cells studied after thawing and culturing. In 16 (73%) cases constitutive NF-κB DNA binding activity could be demonstrated, while 6 cases showed no or only minor levels of NF-κB DNA binding activity (representative data are shown in figure 1A and summarized in table I). Supershift experiments performed with 4 different AML cases with antibodies specific for the p50 and p65 subunits of the NF-κB family indicated that the NF-κB complexes consisted of these subunits (fig. 1B). The constitutive NF-κB DNA binding activity could not be correlated with distinct AML FAB classifications.

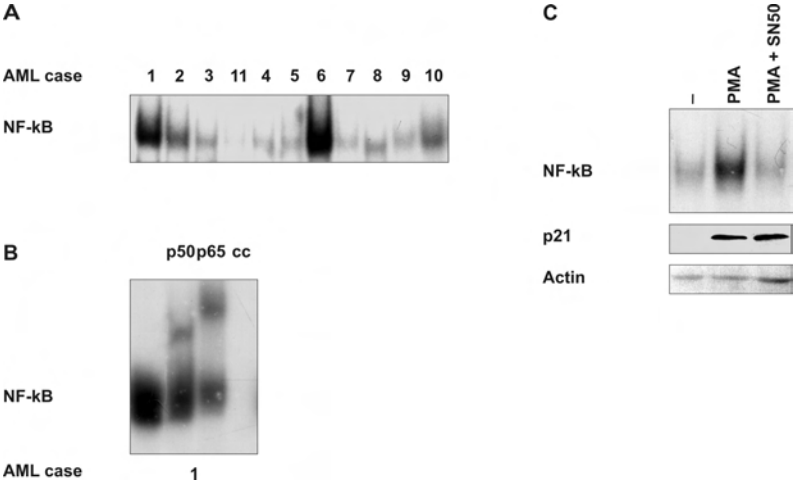

Figure 1 Constitutive NF-κB DNA binding activity in AML blasts.
(A) AML cells were cultured for 24 hours in RPMI 1640 medium with 10% FBS. Nuclear extracts were prepared according to the mini-scale procedure, normalized for protein content and NF-κB DNA binding activity was determined by Electrophoresis mobility shift assay (EMSA), using double-stranded synthetic oligonucleotide probes containing the NF-κB (NF-κB: 5'-AGCTGCGGGGATTTTCCCTG-3') consensus sequence (underlined). (Representative data from 11 AML cases are shown). (B) Supershift experiments were performed by incubating the nuclear extracts with polyclonal antibodies against p50 and p65 subunits of NF-κB. Slower

migrating bands are indicative for the presence of that particular subunit in the complex and thus demonstrate the composition of the NF-κB complex. The specificity of the NF-κB DNA binding was performed with a cold competition assay (cc) as described in materials and methods. (1 representative case is shown) (C) The NF-κB inhibitor SN-50 specifically inhibits NF-κB DNA binding activity. Monocytes were stimulated with PMA after being preincubated with medium or SN-50. NF-κB activity was studied by EMSA and in order to confirm specificity, total p21 expression was analysed by Western blotting. Actin was used as loading control. Densitometric analysis (using the gelscan program Quantity One (Bio-Rad, Hercules, CA, USA)) revealed a 3-fold inhibition of NF-κB DNA binding activity after SN-50 treatment (Data not shown).

Low spontaneous apoptosis of AML blasts correlated with constitutive NF-κB DNA binding activity

Since it has been described that NF-κB plays an important role in cell survival,[189] we examined whether also for AML cells constitutive NF-κB DNA binding activity had an effect on the viability of the cells. AML cells were cultured for 24 hours in 10% FBS and the percentage of apoptotic cells was determined by Annexin V/PI staining. As is shown in table I, AML cases with distinct NF-κB activity (n = 16) demonstrated a significant lower percentage of apoptotic cells (28 ± 13.9%) versus the 6 cases with no NF-κB activity (54.3 ± 14.9% $p < 0.001$).

In order to confirm that NF-κB DNA binding effectively protects AML blasts against apoptosis, we used a specific NF-κB inhibitor, the SN-50 peptide, that protects nuclear import of NF-κB. To this end, AML cells with high constitutive NF-κB DNA binding activity were incubated with the SN-50 peptide and the percentage of apoptotic cells was determined. Addition of SN-50 (100 μg/ml) enhanced the VP-16 (50 μg/ml) induced apoptosis by 13% ± 5% after 24 hours of culture in 5 of 7 cases. VP-16 is an etoposide that induces apoptosis due to inhibition of the resealing activity of topoisomerase II.[277] The inhibitory effect of SN-50 was specific since SN-50 inhibited the PMA induced NF-κB activity without an effect on the PMA induced p21 expression (fig. 1C).

Constitutive Ras activity in AML blasts

In order to elucidate the underlying mechanism responsible for NF-κB activation, we examined whether N-Ras might be constitutively activated in the AML cases. Constitutive N-Ras activity was examined by using the Ras binding domain (RBD) of Raf fused to glutathione S-transferase as an activation specific probe. Western blotting using N-Ras antibodies was performed to detect the binding of GTP-Ras with GST-RBD. As demonstrated in figure 2 and summarized in table I, 15 AML cases demonstrated constitutive N-Ras activity.

This constitutive Ras activity correlated with constitutive NF-κB DNA binding activity ($p < 0.05$). In addition, the expression level of the N-Ras protein was studied for the different AML cases, demonstrating that the amount of Ras protein varied strongly between the different AML cases. The specificity of the Ras pull-down assay was studied by activating AML cells with and without GM-CSF. Upon stimulation with 15 minutes a strong increase in Ras-GTP was shown (fig. 2B).

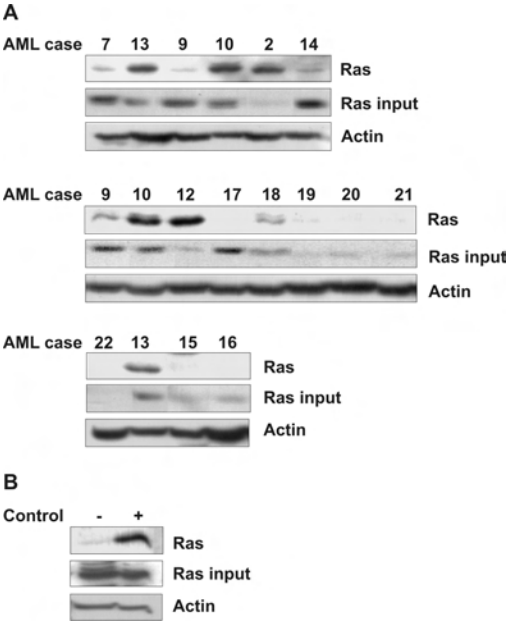

Figure 2 Constitutive Ras activity in AML blasts.
(A) AML blasts were cultured for 24 hours in RPMI 1640 medium with 0.5% FBS. Cell lysates were normalized for protein content and subsequently GTP-bound Ras was precipitated from equal amounts of cell lysates using the Ras binding domain of Raf-GDS fused to glutathione S-transferase as an activation specific probe. The bound proteins were analysed by SDS-PAGE gel and detected by immunoblotting using N-Ras antibody (upper lane). As control for an estimate of the amount of protein used in the pull-down assay, equal amounts of total cell lysates before precipitation were western blotted and immuno-detection was performed using antibodies against N-Ras (Ras input, middle lane) and actin (bottom lane). (Representative data from 15 cases are shown). (B) The specificity of the Ras pull-down assay was studied by activating AML cells with and without GM-CSF (+ and – control respectively) for 15 minutes. Ras-GTP activity was determined as described in A.

The most commonly observed mutations in N-Ras arise at sites critical for gene regulation, namely codons 12, 13, and 61.[266;267] In order to identify the origin of constitutive Ras activity we screened for mutations in codon 12, 13, or 61 of the N-Ras gene by PCR-SSCP analysis and DGGE analysis to identify possible mutations in the K-Ras gene. As shown in table I, only 4 of 22 AML cases demonstrated a mutation in the N-Ras gene, including the TF-1 cell line. Mutations were identified in codon 12 (n=2), 13 (n=1) and 61 (n=2). Furthermore, no mutations were detected in the K-Ras gene.

These data demonstrate that constitutive NF-κB DNA binding activity coincided with constitutive Ras activity, but did not correlate with activating mutations in codon 12, 13, and 61 of the Ras gene.

Constitutive ERK phosphorylation

The classical downstream target of Ras is the Raf/MEK/ERK pathway.[268;270] In addition, some studies have described that a lower percentage of apoptosis in the erytholeukemic TF-1 cell line is associated with higher ERK1/2 activity[278] and the ERK pathway has also been described to be involved in activating NF-κB DNA binding activity.[264;279] Therefore, we investigated whether Ras activity coincided with constitutive ERK activity (Western blots not shown). As summarized in table I, constitutive ERK activity was observed in 17 of 22 AML cases. Although in 12 out of these 17 cases constitutive ERK activity corresponded with constitutive Ras activity, this correlation was demonstrated not be significant.

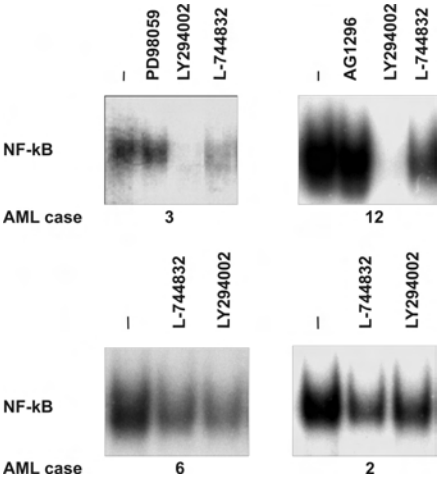

Figure 3 L-744832 and Ly294002 inhibit NF-κB DNA binding activity.
AML cells were cultured for 24 hours in RPMI 1640 medium with 10% FBS supplemented with or without L-744832 (50μM, Ras inhibitor), Ly294002 (5μM, PI3-K inhibitor), AG1296 (20μM, Tyrosine kinase inhibitor) or PD98059 (10μM, MEK1/2 inhibitor). Nuclear extracts were prepared, normalized for protein content and NF-κB DNA binding activity was determined by EMSA. Four representative cases are shown.

L-744832 and Ly294002 inhibit NF-κB DNA binding

In order to investigate the link between Ras and NF-κB in more detail and to elucidate the downstream effectors of Ras facilitating NF-κB activity, we examined the effect of chemical inhibitors, specific for different downstream targets of the Ras pathway, on constitutive NF-κB DNA binding activity. AML blasts were cultured with or without addition of L-744832 (a farnesyl-transferase inhibitor, which blocks Ras activation), PD98059 (MEK1/2 inhibitor), Ly294002 (PI3-K inhibitor), and AG1296 (tyrosine kinase inhibitor). The concentrations used were able to block the respective kinases. In 5 of 7 cases tested the Ras inhibitor L-744832 and the PI3-K inhibitor Ly294002 were able to inhibit constitutive NF-κB DNA binding activity, while AG1296 and PD98059 had no or limited effect. The results of 4 different cases are depicted in figure 3.

These data demonstrate that the ERK kinase, the classical downstream effector of Ras, is not involved in the constitutive NF-κB DNA binding activity. In contrast, both L-744832 and Ly294002 inhibited constitutive NF-κB DNA binding activity, suggesting that Ras might mediate NF-κB DNA binding activity by a PI3-K mediated pathway.

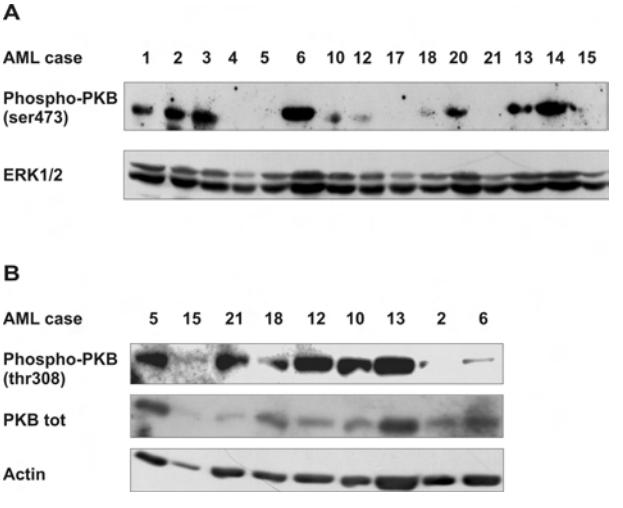

Figure 4 Constitutive PKB phosphorylation in AML.
(A) AML cells were cultured for 24 hours in RPMI 1640 medium with 10% FBS. Total cell extracts were prepared and equal amounts of cell lysates were subjected to SDS-PAGE. Western blot analysis was performed using an antibody against phospho-PKB (Ser473). As a control, equal amounts of total cell lysates were western blotted and immuno-detection was performed using antibodies against total ERK1/2. (Representative data from 15 cases are shown). (B) Cell lysates of AML cases with and without PKB Ser473 phosphorylation were western blotted and subjected to immuno-detection using antibodies against phospho-PKB (Thr308). Total PKB (PKB tot) and actin immuno-detection were performed for loading control. 9 representative cases are shown.

Constitutive PKB phosphorylation in AML

The classical downstream target of PI3-K is PKB/Akt, a protein which is known to prevent apoptosis.[280] Recently, several studies described the involvement of PKB in activation of IKK, the upstream IκB kinase.[281,282] Therefore, we examined whether PKB is constitutively activated in those AML cases that demonstrate constitutive NF-κB activity. In 14 of 22 investigated AML cases, constitutive PKB serine 473 phosphorylation correlated with constitutive NF-κB DNA binding activity ($p < 0.05$, fig. 4A, table I). Since activation of PKB not only involves phosphorylation at the serine 473 residue, but also phosphorylation at the threonine 308 residue,[283] 9 AML samples with and without serine 473 phosphorylation were also investigated for threonine 308 phosphorylation. As is shown in figure 4B, almost all AML samples exhibited threonine 308 phosphorylation, thus leaving phosphorylation of serine 473 a good indicator of activated PKB. Although in 11 cases constitutive PKB activity coincided with

both constitutive Ras activity and NF-κB DNA binding, no significant correlation could be found between Ras and PKB activity.

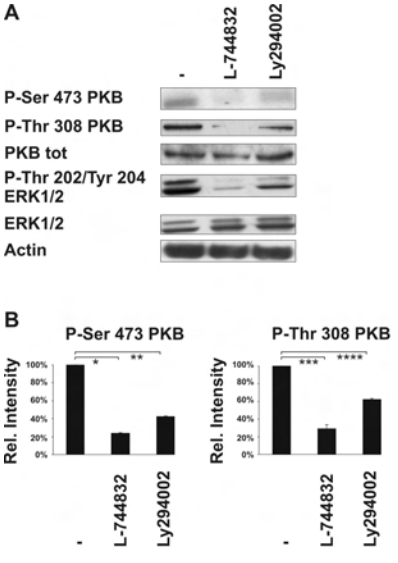

Figure 5 L-744832 and Ly294002 inhibit constitutive PKB phosphorylation in AML.
(A) AML cells were cultured for 24 hours in RPMI 1640 medium with 10% FBS supplemented with or without L-744832 (50μM, Ras inhibitor) or Ly294002 (5μM, PI3-K inhibitor). Total cell extracts were prepared and equal amounts of cell lysates were subjected to SDS-PAGE. Western blot analysis was performed using an antibody against phospho-PKB (Ser473), phospho-PKB (Thr308) and total PKB (PKB tot). In order to show that the L-744832 compound is an effective Ras inhibitor, immuno-detection was performed against on of the downstream effectors of Ras,Phospho Erk1/2 (Thr202/Tyr204). As a control, equal amounts of total cell lysates were subjected to immuno-detection using antibodies against ERK1/2 and actin. The data presented is representative for 4 individual cases. (B) Densitometric analysis was performed on the western blots of P-Ser473 PKB and P-Thr308 PKB using the gelscan program Quantity One (Bio-Rad, Hercules, CA, USA) and normalized against total PKB. Untreated samples were set to 100%. A significant reduction of phosphorylated Ser473 PKB and phosphorylated Thr308 PKB were observed after treatment with the L-744832 and Ly294002 compounds(*, **, *** and **** = $p < 0.0001$). The data presented is averaged from three independent experiments, with error bars denoting standard deviations.

L-744832 and Ly294002 inhibit PKB phosphorylation in AML

In order to elucidate whether the constitutive activation of the PI3-K/PKB pathway in AML is due to constitutive Ras activation, we examined the effect of the Ras inhibitor L-744832 on spontaneous PKB phosphorylation of 4 AML cases with high constitutive Ras, PKB and NF-κB activity. As demonstrated in figure 5A, addition of L-744832 strongly inhibited the spontaneous PKB phosphorylation, both on the serine 473 and the threonine 308 residue. Similarly, addition of Ly294002 strongly reduced PKB phosphorylation on both residues. Western blots for total and phospho-Thr202/Tyr204 ERK1/2 are shown as evidence that the L-744832 compound is effective in inhibiting Ras.
Densitometric analysis showed that this treatment with L-744832 and Ly294002 significantly reduced the levels of serine and threonine phosphorylated PKB (*, **, *** and **** $p < 0.0001$, fig. 5B). The inhibition of Ras and PKB was also reflected at the level of NF-κB DNA binding (fig. 3). Both inhibition of Ras by L-744832 or PKB by Ly294002 resulted in a strong reduction of NF-κB DNA binding activity.

Constitutive NF-κB activity is not caused by autocrine growth factor production

To study whether autocrine growth factor production by AML blasts was responsible for NF-κB activation, a subset of AML blasts with high constitutive NF-κB and Ras activity (no. 2, 6, 10, 5) were cultured for 24 hours in 10% FBS and cell-free supernatant was collected. Subsequently, TF-1 cells, which are highly responsive for IL-1, IL-6, TNFα and GM-CSF, were stimulated for 10 minutes with conditioned AML medium (66%) and the effect on IκB phosphorylation and NF-κB DNA binding activity was examined. As a positive control (to show that IL-1 can actually activate NF-κB in these cells) TF-1 cells were stimulated for 10 minutes with 10 ng/ml IL-1ß. In addition, TNFα also strongly induced NF-κB DNA binding activity in TF-1 cells (data not shown). As shown in figure 6A, none of the AML supernatants induced IκB phosphorylation or distinct NF-κB DNA binding activity, while incubation with IL-1 strongly induced both.

To exclude the possibility that the assay was not sensitive enough, the effect of neutralizing antibodies against IL-1, IL-6, GM-CSF and TNFα on the constitutive NF-κB DNA binding activity of 4 different AML cases was examined. Although the antibodies were capable of inhibiting the cytokine induced Erk phosphorylation (data not shown), after 24 hours of cell culture no significant effect was observed on NF-κB DNA binding activity by any of these neutralizing antibodies (two representative examples are shown in fig. 6B).

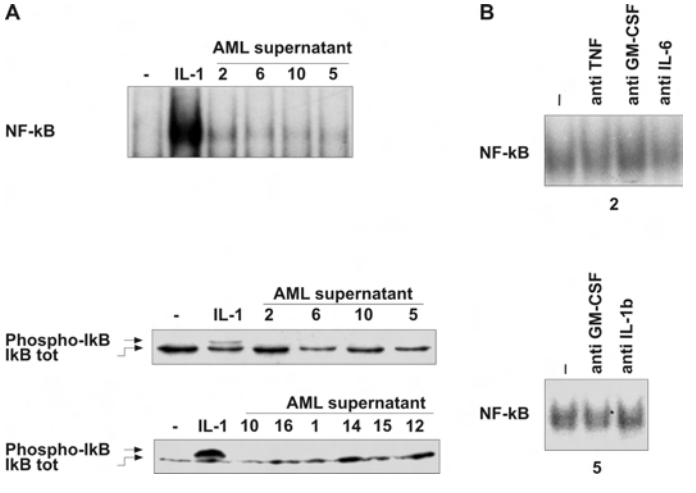

Figure 6 Constitutive NF-κB DNA binding activity is not due to autocrine growth factor production.
(A) In order to investigate whether an autocrine produced growth factor is responsible for NF-κB DNA binding activity, TF-1 cells were cultured in 1% FBS overnight and subsequently stimulated for 10 minutes with the conditioned medium of the indicated AML cases or IL-1β (10 ng/ml), as described in the results. Nuclear extracts were prepared, normalized for the amount of protein and NF-κB DNA binding activity was determined by EMSA (upper panel). Total cell extracts were prepared and equal amounts of cell lysates were subjected to SDS-PAGE. Western blot analysis was performed using an antibody against phospho Ser32-IκB-α and total IκB-α (middle and lower panel). (B) AML blasts were cultured for 24 hours in RPMI 1640 medium with 10% FBS supplemented with or without neutralizing antibodies against IL-1β, IL-6, GM-CSF or TNFα to exclude the possibility that the assay was not sensitive enough. Nuclear extracts were prepared, normalized for protein content and NF-κB binding activity was determined by EMSA (two representative examples are shown).

Constitutive NF-κB DNA binding activity is not due to Flt3 activity

Recently, several reports demonstrated a significant role for the Flt3 tyrosine kinase receptor in the resistance of AML blasts against apoptosis.[40;276] In addition, internal tandem duplication (ITD) mutations in this receptor result in constitutive activation of the receptor and the subsequent activation of several downstream signal transduction cascades, e.g. STAT5 and ERK pathway.[235] Therefore, we investigated whether constitutive NF-κB activity might be due to activation of the Flt3 receptor. First, we examined whether activation of the Flt3 receptor by its ligand could activate NF-κB DNA binding activity. Stimulation of THP-1 cells with Flt3 ligand induced a distinct increase in NF-κB DNA binding activity (fig.7A). In our subset of AML cases, 10 of 22 investigated AML cases demonstrated an internal tandem duplication in the Flt3 gene (see table I). 8 of the 10 cases with an ITD demonstrated constitutive activation of the receptor, while 4 cases demonstrated constitutive Flt3 activation without a mutation (table I). It appeared that in 9 of 12 cases constitutive Flt3 phosphorylation coincided with constitutive NF-κB DNA binding activity. However, addition of the Flt3 inhibitor AG1296 had no effect on the constitutive NF-κB DNA binding activity of the investigated AML blasts (two representative samples are shown in fig.7B), but inhibited significantly the Flt3 mediated STAT5 A/B tyrosine 694/699 phosphorylation as depicted in figure 7B.

In conclusion, Flt3 receptor activation does not account for constitutive NF-κB activity.

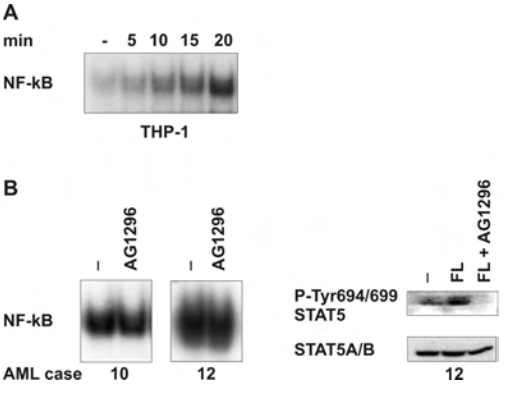

Figure 7 Constitutive NF-κB DNA binding activity is not due to Flt3 activity.
(A) THP-1 cells were cultured overnight in 1% FBS and subsequently stimulated with Flt3 ligand for the indicated periods of time in order to test whether activation of the Flt3 receptor by its ligand could induce NF-κB DNA binding activity. Nuclear extracts were prepared, normalized for protein content and NF-κB binding activity was determined by EMSA. (B) AML blasts were cultured for 24 hours in RPMI 1640 medium with 10% FBS supplemented with or without AG1296 (20μM, Flt3 inhibitor). Nuclear extracts were prepared, normalized for protein content and NF-κB binding activity was determined by EMSA. Although the used concentration of AG1296 (20μM) did not inhibit NF-κB DNA binding activity, it was effective since it inhibited the Flt3 (FL) mediated STAT5A/B Tyr694/699 phosphorylation. As loading control total STAT5 A/B is shown.

Discussion

Several lines of evidence suggest that NF-κB family members are involved in tumor growth.[189] Hodgkin's lymphoma cell lines contain constitutively active NF-κB and inhibition of NF-κB by overexpression of a non-degradable IκBα molecule inhibits proliferation and tumorigenesis of these cells.[284] In addition, activation of NF-κB by tumor necrosis factor, ionizing radiation and chemotherapeutic agents was found to protect tumor cells from cell killing.[285;286] Conversely, inhibition of this transcription factor enhanced apoptotic killing by these reagents, demonstrating that persistent activation of NF-κB represents a unique mechanism by which cells express genes that can protect against apoptotic stimuli.

In the present study we demonstrate that of the investigated AML cases NF-κB was frequently constitutively activated. This constitutive NF-κB activity was associated with a lower percentage of spontaneous apoptosis, compared to AML cases that had no or low nuclear NF-κB expression. In addition, the expression is in general associated with a reduced sensitivity for chemotherapy induced apoptosis.

Selective targeting of leukemic cells through inhibition of NF-κB might be an effective way to enhance the therapeutic effect of existing AML treatment modalities. However, the most rational approach to inhibit NF-κB activity may require the identification of upstream activators. The Ras protein has been shown to be frequently mutated in AML, resulting in the constitutive activity of this GTP-ase,[266;267] and is linked with cellular transformation. Here, we

demonstrate that Ras activation was significant correlated with the NF-κB DNA binding activity in AML blasts. In 68% of the investigated AML cases constitutive N-Ras activity was observed, which was in 27% of the cases associated with mutations in the N-Ras gene. The difference in Ras-GTP activity and the frequency of Ras mutations indicate that alternative causes are also of relevance. Recently a mutation was identified in a Ras-guanine exchange factor in an AML case.[287] Whether these mutations or unidentified growth factors are responsible for the Ras activity will require further studies.
The finding that Ras activation is much more frequent than Ras-mutation is of interest and suggests that the in-vivo application of Ras inhibitors should not be restricted to patients with a mutated Ras gene but that an effect can also be expected in a much larger group of patients.

The classical downstream target of Ras is the Raf/MEK/ERK pathway.[268;270] Several studies described involvement of the ERK pathway in activating NF-κB DNA binding and transactivation.[264;279] In addition, inhibition of the MAPK pathway by several MEK inhibitors resulted in impaired cell growth and survival of acute myeloid leukemia cell lines and primary AML samples with constitutive MAPK activation.[288] However, although ERK was frequently constitutively activated in the AML cases, we did not find any effect of the MEK inhibitor PD98059 on NF-κB DNA binding activity which doesn't exclude the possibility that Erk might effect the NF-κB transactivation potential.[264] In contrast, we demonstrate that Ras mostly mediates NF-κB activation by a PI3-K/PKB dependent pathway. In a number of the cases with constitutive NF-κB DNA binding activity both Ras and PKB were constitutively activated. The link between Ras and the PI3-K/PKB pathway was further confirmed by the fact that the farnesyl-transferase inhibitor L-744832 strongly inhibited PKB phosphorylation. In addition, L-744832 and Ly294002 both strongly inhibited NF-κB DNA binding activity. These data implicate that a Ras/PI3-K/PKB-mediated pathway can mediate constitutive NF-κB DNA binding activity. In addition, it appeared that the constitutive NF-κB DNA binding activity was unlikely caused by production of autocrine growth factors.[289] Furthermore, although aberrant IKK signaling in AML has been described to lead to increased NF-kappaB activity,[215] this could not be detected in the present study, since AML supernatants did not induce IκB phosphorylation. Also the frequently mutated and constitutively activated Flt3 receptor tyrosine kinase was not responsible for NF-κB activation. Therefore, an alternative mechanism, upstream of Ras, is responsible for the constitutive NF-κB activity. We speculate that a yet unknown GTP exchange factor, upstream kinase, or

receptor is mutated resulting in constitutive activation of this signal transduction pathway.

Our results and other findings indicate that a number of different signaling routes are triggered in AML cells with distinct and overlapping activities, including the STAT3 and STAT5 pathways,[40;47] p21 expression in monocytic blasts[182-184;290] and the RAS/PI3-K/NF-κB pathway in these experiments as well as by other investigations.[64;276;284;291] In some cases, activation of these pathways might be triggered by the autocrine production of growth factors and hence lead to enhanced survival and proliferation of the AML blast.[40;47;290;292;293] These findings are in line with the 'two-hit' model whereby two or more triggers are required for cellular transformation. The data also implicate that only a limited success will be obtained if a single inhibitor is used that targets only a small selection of genes.

In summary, our study demonstrates that two crucial anti-apoptotic signaling molecules, Ras and PKB, interfere with the NF-κB pathway and result in constitutive activation of this transcription factor which confers increased survival to AML blasts.

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Constitutive activation of NF-κB is not sufficient to disturb normal steady-state hematopoiesis

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Chapter 3

Abstract

Since NF-κB is frequently activated in AML, we questioned whether active NF-κB affected the cellular properties of cord blood (CB) $CD34^+$ cells. The results demonstrated that NF-κB activation did not influence growth or differentiation properties. Furthermore, NF-κB activation was not sufficient to induce changes in stem- and progenitor cell numbers.

Constitutive activation of Nuclear Factor-κ B (NF-κB) has been observed in a number of patients with Acute Myeloid Leukemia (AML).[294] Predominantly in the myelomonocytic and monocytic subtypes,[215] in contrast to normal $CD34^+$ cells.[64] With a multiplicity of signal transduction pathways converging on NF-κB, this protein is therefore suggested to play a relevant role in disturbed hematopoiesis in AML. Various reports have indicated that constitutive activation of NF-κB is sufficient to induce cellular transformation,[295;296] however, limited information is available on the role of NF-κB in regulating normal hematopoiesis.[297;298] Its role in primary hematopoietic cells is based mainly on inactivation studies using chemical inhibitors, gene knock-outs or overexpression of dominant negative constructs.[297;298] Furthermore, these studies largely focused on the anti-apoptotic properties of NF-κB, where effects on other important cell-biological characteristics such as proliferation, differentiation and self-renewal were underexposed. Therefore, we established a model in which influences were studied of constitutive NF-κB activation on hematopoiesis of primary human $CD34^+$ cells.

Transfection experiments in 293T HEK cells demonstrated that overexpression of wt p65 or a constitutive active IKK-2 (IKK2SE), a kinase upstream of NF-κB,[200] is sufficient to activate an NF-κB responsive luciferase reporter construct (fig. 1A) as compared to control-transfected cells (MIGR1). A TF-1 cell-line, stably transfected with p65, demonstrated enhanced DNA-binding of p65 in EMSAs as well as enhanced NF-κB luciferase reporter activity (2.6-fold for p65 compared to mock transfected cells, data not shown). In addition, Q-PCR for the NF-κB target gene IL-8 demonstrated a 12-fold increase in IL-8 mRNA expression in p65-expressing TF-1 cells (data not shown). Together these data demonstrated that overexpression of either p65 or IKK2SE resulted in an activated NF-κB signal transduction pathway.

Long term co-cultures of transduced cord blood (CB)-derived $CD34^+$ stem- and progenitor cells on MS-5 stromal cell layers indicated no proliferative advantage for p65 or IKK2SE-transduced cells as demonstrated by stable GFP percentages (fig. 1B, dotted lines) compared to MIGR1 transfected cells. Cumulative cell counts from these cultures gave comparable results (fig. 1B, solid lines). Parallel flow-cytometric analysis demonstrated no significant changes in the percentage of GFP^+ cells positive for the myeloid differentiation markers CD11b, CD14, CD15 and CD36, when compared to either GFP^- cells within the same culture or to MIGR1 transfected cells (N=3, a representative example is shown in fig. 1C).

In addition, progenitors were enumerated in CFC assays in limiting dilution. IKK2SE transduction demonstrated no significant advantage compared to

MIGR transduction in progenitor frequencies (14.5% vs. 13.7% respectively, data not shown). In separate CFU-GM and BFU-E assays also no differences were observed (data not shown).

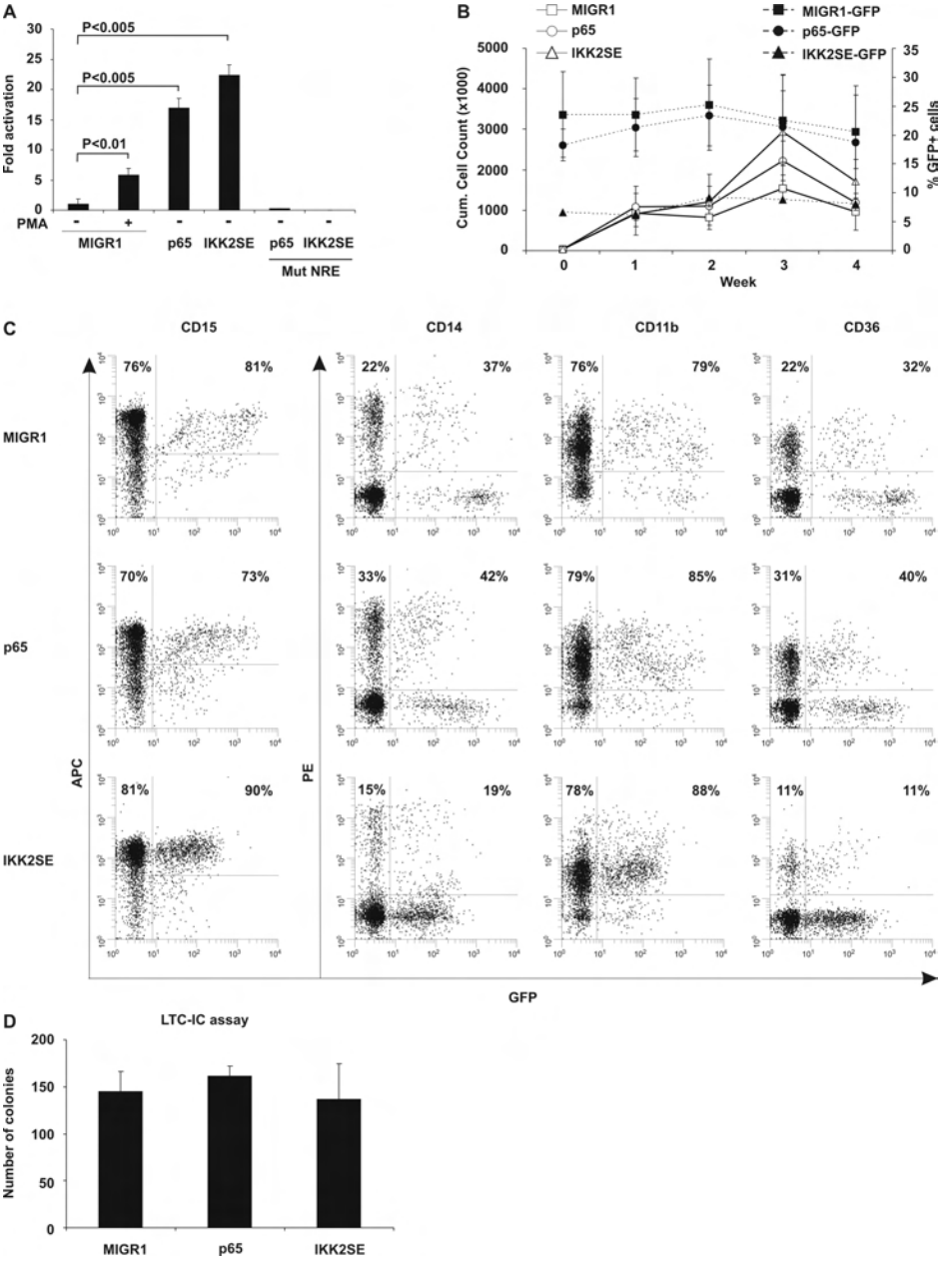

Figure 1 NF-κB activation does not alter growth, differentiation or self-renewal capacity of cord blood (CB) CD34$^+$ cells on MS-5 long-term co-cultures.
(A) 293T HEK cells were co-transfected with MIGR1, p65 or IKK2SE mutant constructs together with a luciferase vector containing 3 NF-κB responsive elements (NREs). PMA-stimulated (50 nM, 16 hrs) cells were used as positive control and Mut NRE (in which the 3 NREs are mutated)-luciferase vector transfected cells as negative control. Experiments were performed in triplicate (N=3). A representative example is shown. Data is presented as average with standard error of the mean (SEM) and a student's T-test was performed to calculate significance levels. (B) MS5 co-culture experiment with MIGR1, p65 and IKK2SE transduced CB CD34$^+$ cells. Dotted lines indicate GFP percentages, with errors bars representing SEM, (N = 3). Solid lines indicate cumulative cell counts. An average of 3 independent experiments is shown. Error bars represent SEM. (C) FACS analysis of a representative week 3 MS5 co-culture experiment with MIGR1, p65 or IKK2SE transduced CB CD34$^+$ cells. Shown are percentages of CD15, CD14, CD11b and CD36 positive cells in either untransduced cells or in transduced GFP$^+$ cells, (N = 3). (D) LTC-IC assay with MIGR1, p65 or IKK2SE transduced CB CD34$^+$ cells. After 5 weeks on MS5 co-culture, medium was changed to methylcellulose for another two weeks. At week 7 colonies were scored. An average of 3 independent experiments is shown. Error bars denote SEM.

In order to investigate whether active NF-κB affected the self-renewal capacity of hematopoietic stem cells, LTC-IC assays were performed with MIGR1-, p65- and IKK2SE-transduced CB CD34$^+$ cells, by adding methyl-cellulose to a week 5 MS-5 co-culture (as described previously)[244] and determining the number of colonies at week 7. No significant difference in colony formation between p65-, IKK2SE- or MIGR1-transduced cells was detected (fig. 1D). Together these findings indicate that in human CD34$^+$ cells, constitutively activation of NF-κB is not sufficient to change their differentiation potential. Others have shown that a reduction of NF-κB activity in murine fetal liver cells or ES-derived hematopoietic progenitors results in severely disturbed myeloid differentiation.[297;298] Apparently NF-κB activation is required for normal myelopoiesis, but its increased activity is not sufficient to impair differentiation.

In normal and leukemic stem and progenitor cells it has been demonstrated that NF-κB antagonizes (ROS-mediated) apoptosis.[64;298] In contrast, we did not detect a reduced level of apoptosis (by Annexin V staining, data not shown) in p65 or IKK2SE transfected CB CD34$^+$ cells compared to control cultures. Additionally, IL-3 deprivation of p65 expressing stable TF-1 cell lines did not show reduced apoptosis (data not shown). This is in line with data from Romano et al,[299] demonstrating that constitutive NF-κB activity is not relevant for sustained basal cell survival of CB CD34$^+$ and AML cells, but only when the cells are triggered with a stress response, e.g. exposure to chemotherapy.

Together these data demonstrate that constitutive activation of NF-κB as a single "hit" is not sufficient to induce changes in steady-state hematopoiesis with regard to proliferation, differentiation, self renewal and apoptosis, which could potentially bias cells towards a more leukemogenic phenotype. Whether constitutive NF-κB activation in concert with additional triggers has alternative

functions on hematopoiesis needs to be further defined, in order to gain insight in their respective and potentially additive roles in the events leading towards AML.

Acknowledgements

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Constitutive cytoplasmic localization of $p21^{Waf1/Cip1}$ affects the apoptotic process in monocytic leukemia

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Chapter 4

Abstract

In the present study, we analysed the expression and localization of $p21^{Waf1/Cip1}$ in normal and malignant hematopoietic cells. We demonstrate that in normal monocytic cells, protein kinase C (PKC)-induced p21 gene activation, which is Nuclear Factor-κB (NF-κB) independent, results in predominantly cytoplasmic localized p21 protein. In acute monocytic leukemia (M4, M5), monocytic blasts (N=12) show constitutive cytoplasmic p21 expression in 75% of the cases, while in myeloid leukemic blasts (N=10) low nuclear and cytoplasmic localization of p21 could be detected, which is also PKC dependent. Constitutive p21 expression in monocytic leukemia might have important anti-apoptotic functions. This is supported by the finding that in U937 cells overexpressing p21, VP16-induced apoptosis is significantly reduced (20.0% ± 0.9 vs. 55.8% ± 3.8, $p<0.01$, N=5), reflected by a reduced phosphorylation of p38 and JNK. Similar, AML blasts with high cytoplasmic p21 were less sensitive to VP16-induced apoptosis as compared to AML cases with low or undetectable p21 expression (42.25% vs. 12.3%, $p<0.01$). Moreover, complex formation between p21 and ASK1 could be demonstrated in AML cells by means of co-immunoprecipitation. In summary, these results indicate that p21 has an anti-apoptotic role in monocytic leukemia and p21 expression is regulated in a PKC-dependent and NF-κB-independent manner.

Introduction

Acute myeloid leukemia (AML) is characterized by an accumulation of immature cells in the bone marrow resulting in the disruption of normal hematopoiesis.[16;17;300] The growth advantage of the leukemic population is in part linked to the constitutive activation of intracellular proteins that trigger the function of anti-apoptotic proteins.[40;64] Recent studies have indicated that Nuclear Factor-κB (NF-κB) is constitutively expressed in AML cells which is due to the activation of the Ras/PI3 kinase pathway.[64;276;284;291] A recent study in normal monocytes has suggested that the anti-apoptotic function of NF-κB is in part mediated by cytoplasmic p21$^{Waf1/Cip1}$ (p21) protein.[182;183] So far p21, a member of the family of cyclin-dependent kinase (CDK) inhibitors, was defined as a nuclear protein. These proteins function by inhibiting CDK1, -2, -4 and -6 and induce cell cycle arrest at the G1/S boundary. This process allows the cells to exit the cell cycle and differentiate.[301-306] Localization of p21 in the cytoplasm has been connected to monocytic differentiation and is implicated in anti-apoptotic functions.[182-184] Cytoplasmic p21 can form a complex with apoptosis signal regulating kinase 1 (ASK1), preventing the activation of the mitogen activated protein (MAP) kinase cascade.[182] In addition p21 can bind to pro-caspase 3, preventing the induction of apoptosis in HepG2 cells.[307;308] Finally, p21 has been shown to be a downstream target of protein kinase B (PKB (AKT)). Two sites in the carboxyl terminus of p21 are phosphorylated by PKB which enhance the stability of the p21 protein and modulate binding of p21 to target proteins.[309;310]

In view of the link between p21 and apoptosis we questioned whether p21 expression and localization is perturbed in malignant hematopoietic cells and whether cytoplasmic localization of p21 protects against the cytotoxic effects of chemotherapy. The results demonstrate that p21 is not expressed in unstimulated hematopoietic cells of monocytic and myeloid origin. In addition it is demonstrated that PMA is capable of inducing p21 expression in a protein kinase C (PKC) dependent manner. In monocytic leukemia, p21 is constitutively expressed and cytoplasmic localized, in contrast to myeloid leukemia where no cytoplasmic p21 was detected. We postulate that cytoplasmic p21 protects these cells against the effects of cytotoxic agents, as is reflected by a decrease in VP16-induced apoptosis in p21 overexpressing cells and AML blasts with constitutive p21 expression.

Materials and methods

Patient population and isolation of AML cells - Peripheral blood cells or bone marrow cells from 22 adult untreated patients with AML were studied after informed consent. The AML cases were defined according to the classification of the French-American-British (FAB) committee as M0-M6.[18] AML blasts were isolated by density-gradient centrifugation. The cells were cryopreserved in aliquots of 20-30 x 10^6 cells in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS; Bodinco, Alkmaar, the Netherlands), employing a method of controlled freezing and storage in liquid nitrogen. After thawing, T-lymphocytes were depleted by 2-aminoethylisothioronium bromide (AET)-treated sheep red blood cell (SRBC) rosetting. The cell population consisted of more than 98% AML blasts as determined by May-Grünwald-Giemsa staining. Fluorescence-activated cell sorting (FACS) analysis demonstrated <1% CD3 (Becton Dickinson, Sunnyvale, California, USA) positive cells.

Preparation of monocytes and granulocytes - Peripheral blood cells were obtained from healthy volunteer blood donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque density-gradient centrifugation. T- lymphocytes were depleted by AET-treated SRBC rosetting. Monocytes were further enriched by plastic adherence (1h, 37°C, 5% CO_2) and demonstrated a purity >95% detected by FACS analysis with anti-CD14 antibody (Becton Dickinson, Sunnyvale, CA).

Peripheral blood from healthy volunteers, anti-coagulated with 0.32% sodium citrate, was used to isolate granulocytes as described by Koenderman et al.[311] In short, mononuclear cells were removed by centrifugation over Fycoll-Paque (Amersham, Upsala, Sweden) and erythrocytes were lysed with ice-cold NH_4Cl solution. Granulocytes were allowed to recover for 30 min at 37°C in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 0.5 % human serum albumin (HSA; CLB, Amsterdam, The Netherlands). Before stimulation, cells were resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$, 0.5% HSA). In all cases, the cell population isolated consisted of >95% granulocytes as determined by May-Grünwald Giemsa staining.

Cell culture - AML blasts were cultured at 37°C, 5% CO_2 at a density of $1x10^6$/ml in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (ICN, Costa Mesa, CA, USA) and 10% FBS. Monocytes

were cultured at 37°C, 5% CO_2 at density of 1 x 10^6 /ml in RPMI 1640 and 10% FBS. The human growth factor dependent cell lines AS-E2 [312] and GF-D8 [313]were cultured in RPMI 1640 supplemented with 5% FBS and 10 ng/ml IL-3 (Genetics Institute Cambridge, MA). The human cell lines U937 (ATCC, Product No. CRL-1593.2), THP-1 (ATCC, Product No. TIB-202) and HL-60 (ATCC, Product No. CCL-240) were cultured in RPMI 1640 supplemented with 10% FBS. The primitive human cell line KG1a (ATCC, Product No. CCL-2461) was cultured in IMDM (ICN) with 20% FBS. In addition U937 cells stable transfected with a $ZnCl_2$ (180 µM, 3 days) inducible p21 construct were used in conjunction with a mock transfected cell line for studying the cellular function of p21.[182;184]

Reagents and antibodies - Antibodies against phosphorylated Extra-cellular-signal Regulated Kinase (ERK), Protein Kinase B (PKB), p38 and c-jun N-terminal kinase (JNK) were obtained from New England Biolabs (Beverly, MA, USA). Antibodies against p38, JNK, ASK1 and an antibody-agarose conjugate for immunoprecipitation of p21 (C-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p21 antibody was purchased from Transduction Laboratories (Lexington, KY, USA), and an antibody against actin was obtained from Boehringer Mannheim (Mannheim, Germany). Supershift antibodies for p50 and p65 were purchased from Santa Cruz Biotechnology, as well as the Rb antibody. Horse radish peroxidase (HRP)-labeled secondary antibodies were obtained from DAKO (Glostrup, Denmark).
Recombinant human (Rh) interleukin (IL)-1ß was obtained from Mekesson HBOC Bioservices (Rockville, MD, USA). Rh IL-3 and Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) were purchased from Genetics Institute (Cambridge, MA, USA) and phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma (St. Louis, MO, USA) as well as Actinomycin D. The p38 MAPK inhibitor SB203580 and the NF-κB inhibitor SN-50 were obtained from Biomol (Plymouth Meeting, PA, USA). The PI3K inhibitor Ly294002 was obtained from Alexis (San Diego, CA, USA). MAP kinase/ERK kinase (MEK) 1 inhibitor U0126 was obtained from Promega Corp. (Leiden, the Netherlands). The PKC inhibitors Bisindolylmaleimide I (BIM or GF 109203X), Chelerythrin and Calphostin-C were obtained from Calbiochem (Darmstadt, Germany). VP16 was purchased from TEVA Pharming (Mijdrecht, The Netherlands). Radio-nucleotides and the random hexamer primers p(CNA)6 were obtained from Amersham (Buckinghamshire, UK). H_2O_2 was obtained from Merck (Darmstadt, Germany). M-MLV-RT polymerase and RNAse inhibitor were obtained from MBI Fermentas (St.Leon-Rot, Germany). Tri Reagent was purchased from MRC Molecular Research Center Inc. (Cincinnati, OH, USA).

The Quantitect SYBR Green PCR Kit was obtained from Qiagen GmbH (Hilden, Germany). The dNTP's were purchased from Invitrogen BV (Breda, The Netherlands).

Combined annexin V/PI staining procedure - Viability was assessed using an annexin V staining kit (IQ Products, Groningen, The Netherlands) according to the manufacturer's recommendations. Briefly, after 24 hrs of culture in RPMI 1640 medium supplemented with 10% FBS with or without addition of VP16 (20 µg/ml), cells were harvested, resuspended in 100 µl calciumbuffer containing 5 µl of annexin V and incubated for 20 min at 4°C in the dark. Cells were washed with 5 ml calciumbuffer and subsequently incubated in 300 µl calciumbuffer containing 2.5 µl of propidium iodide (PI) for 10 min in the dark. Finally, binding of fluorescein-conjugated annexin V and PI was measured by fluorescence-activated cell sorting (Becton Dickinson, Sunnyvale, CA). Apoptosis was also analysed by measuring active caspase 3 levels by flow cytometry using a FITC-labeled antibody (BD Biosciences, Alphen aan den Rijn, The Netherlands) against the active form of caspase 3. The cells were fixed and permeabilized with fix and perm obtained from BD Biosciences.

Preparation of protein extracts and Western blotting - The amount of p21, ASK1, p38, JNK, and actin and the degree of phosphorylated p38 and JNK were determined by western blotting on whole cell extracts, cytoplasmic or nuclear extracts. U937 cell-lines were cultured for 3 days in RPMI 1640 supplemented with 10% FBS, with or without $ZnCl_2$ (180 µM) and overnight with VP16 (20 µg/ml) or H_2O_2 (300 µM). Cells were harvested and total cell extracts were prepared by resuspending the cells in sample buffer (2% SDS, 10% glycerol, 2% ß-mercapthoethanol, 60 mM Tris-HCl pH 6.8 and bromophenol blue). Cell extracts were directly boiled for 5 minutes and proteins were fractionated by running on SDS-PAGE gel. The proteins were electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA, USA) blocked with TBS buffer containing 0.1% Tween-20 and 5% non-fat milk prior to incubation with antibodies, diluted in 5% bovine serum albumin. Binding of each antibody was detected by HRP labeled secondary antibodies using enhanced chemiluminesence (ECL) according to the manufacturers recommendations (Amersham Life Sciences, Buckinghamshire, UK).

Monocytes and AML blasts were cultured for 24 hrs in RPMI 1640 supplemented with 10% FBS with or without PMA (50 nM), IL-1ß (10 ng/ml), IL-3 (10 ng/ml), GM-CSF (10 ng/ml), Ly294002 (5 µM), SB203580 (1 µM), U0126 (5 µM), BIM (1 µM), Chelerythrin (6 µM) or Calphostin-C (100 nM) at a density of $1x10^6$ cells/ml. Cells were harvested and cytoplasmic and nuclear extracts

were prepared according to the “mini extracts” method.[273] The extracts were normalized for protein content prior to SDS-PAGE. Proper fractionation and lack of leakage of nuclear proteins to the cytosol was determined with western blotting against the nuclear protein Retinoblastoma (Rb).

To investigate the role of an autocrine growth factor, AML blast were cultured for 24 hrs in RPMI 1640 supplemented with 10% FBS and subsequently stimulated for 6 hrs with conditioned AML medium (prepared by incubating AML cells at a density of 3.10^6 cells/ml for 3 days) or 50 nM PMA as a control.

RNA extraction, preparation of cDNA and Real-Time PCR - 5 x 10^6 monocytes were cultured for 24 hrs in RPMI 1640 supplemented with 10% FCS and subsequently stimulated for 0, 1, 2 or 3 hrs with 50 nM PMA, in the presence and absence of 1 μg/ml Actinomycin D. Cells were lysed in 1 ml Tri Reagent, and RNA was isolated according to the manufacturers recommendations. Briefly, 0.2 ml chloroform was added to the Tri reagent cell mixture, and after 5 minutes at room temperature the RNA was phase separated into the aqueous phase by centrifuging at 12000 x g for 15 minutes at 4°C. The RNA was precipitated from the aqueous phase by mixing and centrifuging with 1 volume of isopropanol for 10 minutes at 12000 x g at 4°C. After washing the RNA pellet with 1 ml 75% ethanol, it was dried and dissolved in DepC-treated H_2O.
The isolated RNA was used to make cDNA. Briefly, annealing of the random hexamer primers (p(CNA)6) was performed by incubating 5 μl of RNA for 10 minutes at 65°C in the presence of the random hexamer primers, and immediately putting them on ice afterwards.

The synthesizing of the cDNA was then completed by incubating the samples at 37°C for 1 hour in the presence of 0.5 mM dNTP’s, M-MLV-RT polymerase and RNAse inhibitor.
A real-time pcr analysis of p21 expression was performed using the Quantitect SYBR Green PCR Kit from Qiagen, following the manufacturers instructions on the Light Cycler system from Roche. The p21 values were normalised against values obtained by real-time pcr for HPRT. Primers used: p21 FOR: tcaccgagacaccactggag, p21 REV: cttccaggactgcaggcttc. HPRT FOR: tggcgtcgtgattagtgatg, HPRT REV: gatgtaatccagcaggtcag.

Electrophoretic mobility shift assay - Monocytes were cultured for 24 hrs in RPMI 1640 supplemented with 10% FBS, with or without addition of SN-50 (100 μg/ml) and subsequently stimulated for 6 hrs with 50 nM PMA. Nuclear

extracts were prepared according to the “mini extracts” procedure as previously described, divided in small aliquots and stored at -80°C.

Double-stranded synthetic oligonucleotide probes containing the NF-κB (NF-κB: 5'-AGCTGCGGGGATTTTCCCTG-3') consensus binding sequence were used in the gel retardation assay. The consensus sequence for binding of the nuclear factors is underlined. 50 ng of high performance liquid chromatography (HPLC)-purified single-stranded oligonucleotide was labeled with T4-polynucleotide kinase and [α^{32}P] dATP (3000 Ci/mmol), separated from non-incorporated radiolabel by sephadex G50 chromatography, ethanol precipitated, dried, and dissolved in 20 µl of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, containing a four fold excess of the opposite strand. Annealing of the two strands was performed by heating the mixture for 2 minutes at 90°C and slow cooling to room temperature. 5 µg nuclear extract and 0.1 ng double-stranded labeled oligonucleotide were incubated in 20 mM HEPES (pH 7.9), 60 mM KCl, 0.06 mM EDTA, 0.6 mM DTT, 2 mM spermidine, 10 % glycerol, supplemented with 2 µg poly(dI-dC). The binding reaction was performed at 26°C for 25 minutes. The samples were loaded on pre-run (30 min, 150 V) 4% (30:1) polyacrylamide gels and run for 1 hr at 150 V in 0.5 x TBE at room temperature. Gels were dried and exposed to Kodak XAR films at -80°C with an intensifying screen. Quantification of protein binding was performed by densitometry using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Competition experiments were performed by adding a 100-fold molar excess of unlabelled double stranded oligonucleotides. Supershift experiments were performed by incubating the nuclear extracts for 30 minutes with polyclonal antibodies against the p50 and p65 subunits of NF-κB.

Immunoprecipitation - 10^7 AML blasts were cultured for 16 hrs in RPMI 1640 medium supplemented with 10% FBS with or without 50 nM PMA. As positive control 10^7 cells of the human growth factor dependent cell-line AS-E2 were cultured for 16 hrs in RPMI 1640 supplemented with 5% FBS and 10 ng/ml IL-3. These were cultured in the presence of 50 nM PMA to induce p21 expression. Cells were harvested, washed with ice-cold PBS containing 1 mM sodium orthovanadate and subsequently lysed in 500µl lysisbuffer (20 mM TrisHCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 µM pepstatin and 1 mM DTT) for 15 min on ice. Cell lysates were clarified at 10 000g for 20 min and incubated with 10 µl p21 agarose conjugate (p21 (C-19) AC Santa Cruz, CA, USA) rotating O/N at 4°C. The immune complex was washed 3 times with lysisbuffer and heated in

sample buffer, separated by SDS-PAGE, immunoblotted on PVDF membrane (Millipore, Bedford, MA) and incubated with anti-p21 and anti-ASK1 antibodies. Immunocomplexes were detected using ECL (Amersham, UK).

Statistical analysis - The student t-test for paired samples was used to determine statistical significance of the apoptosis data in the U937 cell-line. The Mann-Whitney U test was used to determine statistical significance between the two groups of differentially expressing p21 AML blasts.

Results

Expression and regulation of p21 in normal monocytes and myeloid cells

To obtain more information regarding expression, localization and regulation of p21 in normal hematopoietic cells and especially whether p21 is a downstream target of NF-κB, as is reported earlier,[183] monocytes from healthy donors were stimulated with IL-1ß, GM-CSF, IL-3 and the phorbol ester PMA for 4, 6 and 24 hours. As depicted in fig. 1A, in unstimulated cells no p21 protein could be detected while PMA, IL-3 and GM-CSF all induced p21 expression, with a predominant cytoplasmic localization. Correct fractionation is indicated by the absence of leakage of the nuclear Rb protein to the cytoplasm. Stimulation with IL-1ß did not result in the appearance of p21 protein, despite the fact that IL-1 is capable of inducing NF-κB DNA binding activity.[194;278;293;314-316] Since the appearance of p21 could reflect increased p21 gene expression or a diminished p21 protein degradation,[317] a Real-Time PCR was performed on PMA stimulated monocytes. Treatment of monocytic cells from healthy donors with PMA up to 3 hours clearly showed an upregulation of p21 gene expression, which could effectively be blocked by treatment with the Polymerase II inhibitor Actinomycin D (fig. 1B). This indicates that PMA can regulate p21 at the level of gene expression. Next we determined whether NF-κB is activated by PMA and is required for PMA-induced p21 expression. Monocytes were stimulated with PMA and IL-1 and analysis of DNA binding by NF-κB was performed with an Electrophoretic Mobility Shift Assay (EMSA) as described in material and methods. PMA and IL-1 both induce NF-κB binding to its consensus binding sequence (fig. 1C). Supershift experiments with antibodies directed against the NF-κB subunits p50 and p65 resulted in complexes with a decreased mobility, indicating the presence of NF-κB in the observed complexes. Addition of a molar excess of unlabelled probe resulted in a complete disappearance of IL-1 induced DNA-protein complexes. In order to

analyse if the PMA induced NF-κB activation is involved in the upregulation of p21 expression, monocytes were stimulated with PMA in the absence and presence of the NF-κB inhibitor SN-50. As demonstrated in fig. 1D, SN-50 inhibited the induction of NF-κB DNA binding activity in response to PMA stimulation, but did not affect the PMA-induced p21 expression. These data indicate that in monocytes NF-κB is not required for PMA-induced p21 expression.

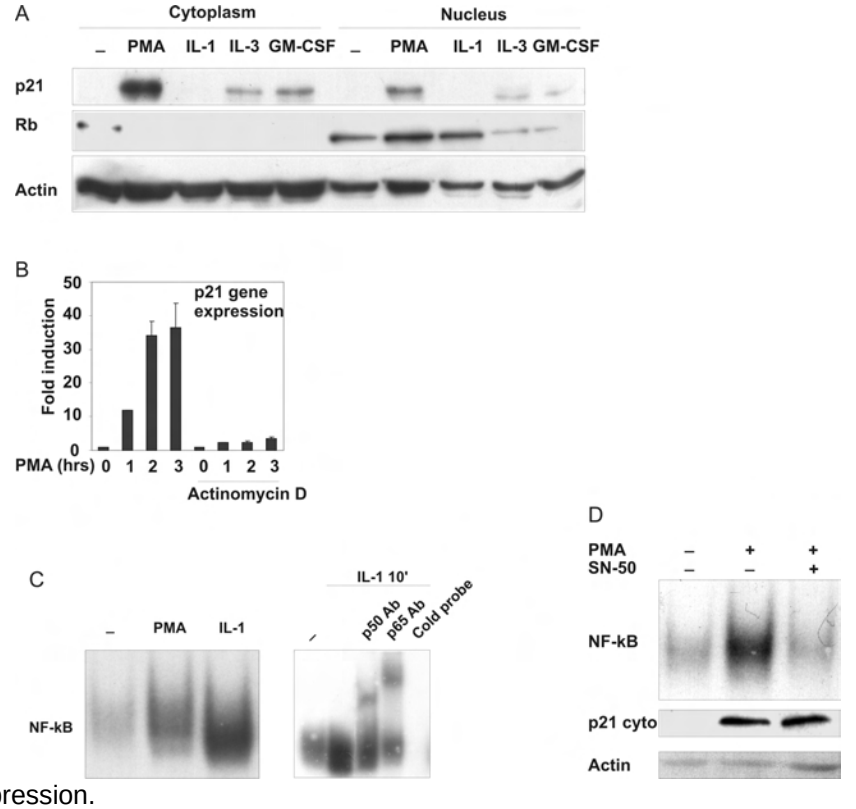

Figure 1 Expression and localisation of p21 in monocytes.
(A) Monocytes from healthy donors were isolated and cultured as described in material and methods, and stimulated with 50 nM PMA, 10 ng/ml IL-1ß, 10 ng/ml IL-3 and 10 ng/ml GM-CSF for 24 hrs. Nuclear and cytoplasmic fractions were analysed by SDS-PAGE. Rb is used as a fractionation control. The data presented are representative for 5 individual experiments. (B) 5 x 10^6 monocytes were cultured for 24 hrs in RPMI 1640 supplemented with 10% FCS and subsequently stimulated for 0, 1, 2 or 3 hrs with 50 nM PMA, in the presence and absence of 1 μg/ml Actinomycin D. RNA was isolated and cDNA was synthesized as described in material and methods. This cDNA was subjected to Real-Time PCR in order to analyse p21 gene expression, and was normalized against HPRT. The data presented is averaged from two individual experiments. (C) Monocytes were cultured for 6 hrs in the presence of 50 nM PMA or 10 ng/ml IL-1ß. EMSA's were used to detect DNA binding activity of NF-κB in nuclear extracts. Controls with α-p50 en α-p65 antibodies for supershifts and cold probe competition are shown for IL-1 treated samples in order to verify that the observed complex is specific and contains NF-κB. The data

presented is representative for 3 individual experiments. (D) After culturing monocytes for 6 hrs in the presence 50 nM PMA with or without pre-treatment with the specific NF-κB inhibitor SN-50 (100 µg/ml), nuclear extracts were analysed for DNA binding capacity of NF-κB in an electrophoretic mobility shift assay. Cytoplasmic extracts were analysed for p21 content by SDS-PAGE. The data presented is representative for 2 individual experiments. Cyto = cytoplasmic.

To further investigate the expression and role of p21 in cells of monocytic and myeloid origin, granulocytes and the cell lines KG1a, HL-60, AS-E2, GF-D8 and U937 were stimulated with GM-CSF or PMA. In none of the unstimulated cell-lines p21 could be detected in the nucleus or cytoplasm. Upon stimulation with PMA during 6 hours, an increase in cytoplasmic and nuclear p21 was demonstrated in all cell-lines studied, which was most prominent in the monocytic cell line U937 (fig. 2A). In the primitive KG1a cell line and in granulocytes, PMA or GM-CSF induced p21 expression was low or undetectable compared to monocytic cells (fig. 2B). These findings indicate that in hematopoietic cells, p21 expression is absent in unstimulated cells, and can be significantly upregulated after induction with PMA, GM-CSF or other cytokines, with the exception of the KG1a cells and granulocytes. In addition, while cells of the myeloid lineage showed comparable amounts of cytoplasmic and nuclear p21, monocytes were observed to have more p21 protein localized in the cytoplasm after PMA and GM-CSF treatment (fig. 1A and 2A).

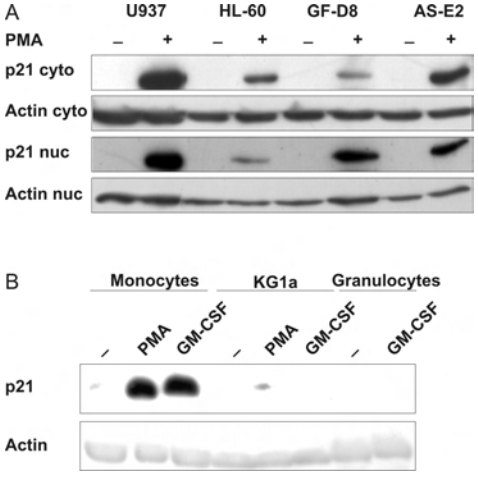

Figure 2 Expression and localisation of p21 in hematopoietic cells.
(A) Expression of p21 protein in U937, HL-60, GF-D8 and AS-E2 cells. After culturing these cells in the presence or absence of 50 nM PMA for 6 hrs, nuclear and cytoplasmic extracts were analysed for p21 levels by SDS-PAGE. The data presented are representative for 2 individual experiments. Cyto = cytoplasmic, nuc = nuclear. (B) Expression of p21 in the primitive cell-line KG1a and granulocytes. Granulocytes were obtained from healthy donors and stimulated with 50 nM PMA or 10 ng/ml GM-CSF for 6 hrs. KG1a cells were stimulated with 50 nM PMA or 10 ng/ml GM-CSF for 6 hrs. Western blot analysis of whole extracts was performed to demonstrate p21 expression upon GM-CSF and PMA treatment. Monocytes stimulated with PMA and GM-CSF were used as positive controls. Actin was used as loading control. The data presented is representative for 4 individual experiments.

Using chemical inhibitors, the role of various intracellular signaling routes in regulating p21 expression in monocytes was investigated. Inhibitors used were: U0126, SB203580 and Ly294002, that block the Map kinase/ERK kinase1

(MEK1), p38 kinase and the PI(3)K activity, respectively. In monocytes, neither U0126, SB203580, nor Ly294002 affected PMA (fig. 3A) and GM-CSF (data not shown) induced p21 expression, while the used inhibitors effectively interfered with the activation of downstream effectors (fig. 3B). The PKC inhibitors BIM and Chelerytrine however strongly inhibited p21 expression. Low concentrations of BIM display activity on typical and atypical PKC isoforms.[318] Similar findings have been described for Chelerytrine.[319] The PKC inhibitor Calphostin-C, which has been described to act on classical and novel PKC isoforms,[319-321] had no effect on p21 expression (fig. 3A). In all cases, localization of the p21 protein was not affected by the use of different inhibitors. The absence of nuclear p21 after treatment with BIM and Chelerythrin is probably due to the lowered expression levels, instead of re-localization. These findings indicate that in monocytic cells, a PKC-dependent pathway is responsible for the expression of the p21 protein, although it remains to be determined which PKC isoform is the main effector of this process.

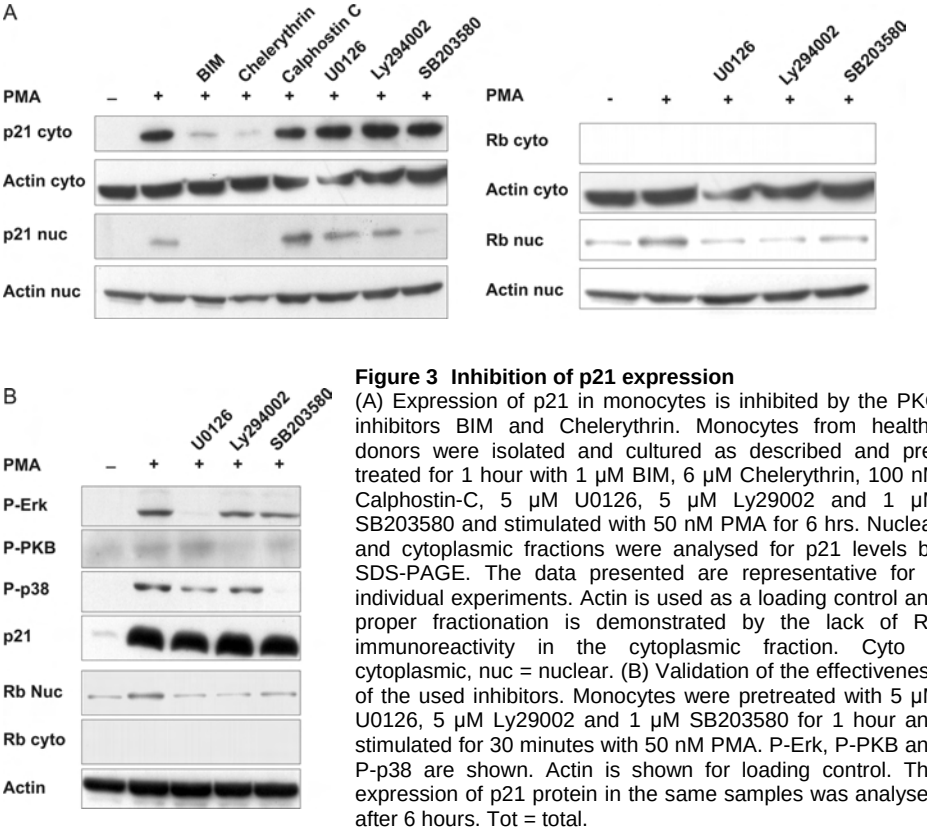

Figure 3 Inhibition of p21 expression
(A) Expression of p21 in monocytes is inhibited by the PKC inhibitors BIM and Chelerythrin. Monocytes from healthy donors were isolated and cultured as described and pre-treated for 1 hour with 1 μM BIM, 6 μM Chelerythrin, 100 nM Calphostin-C, 5 μM U0126, 5 μM Ly29002 and 1 μM SB203580 and stimulated with 50 nM PMA for 6 hrs. Nuclear and cytoplasmic fractions were analysed for p21 levels by SDS-PAGE. The data presented are representative for 3 individual experiments. Actin is used as a loading control and proper fractionation is demonstrated by the lack of Rb immunoreactivity in the cytoplasmic fraction. Cyto = cytoplasmic, nuc = nuclear. (B) Validation of the effectiveness of the used inhibitors. Monocytes were pretreated with 5 μM U0126, 5 μM Ly29002 and 1 μM SB203580 for 1 hour and stimulated for 30 minutes with 50 nM PMA. P-Erk, P-PKB and P-p38 are shown. Actin is shown for loading control. The expression of p21 protein in the same samples was analysed after 6 hours. Tot = total.

p21 expression in Acute Myeloid Leukemia

In hematopoietic cells, p21 is not expressed in unstimulated cells but can be induced by PMA in the monocytic lineage in a PKC-dependent fashion. In order to see if a similar expression pattern exists in the malignant counterpart, p21 was studied in cryopreserved AML cells of either monocytic or myeloid origin. In contrast to normal unstimulated monocytes, a high constitutive expression of p21 was observed in 75% of the monocytic leukemia (M4-5, N=12) with a predominant cytoplasmic localization (fig. 4A). In 50 % of the myeloid leukemia's (M1-2, N=10) p21 expression could not be detected without stimulation, or a low expression of p21 was observed, which was localized in both the nucleus and cytoplasm (fig. 4A and data not shown). These findings link aberrant p21 expression primarily to cells of monocytic origin. To demonstrate whether PKC is also involved in the spontaneous expression of p21 in AML cells, blast cells (N=5) with a high cytoplasmic p21 localization were exposed to the PKC inhibitors BIM and Chelerythrin. Chelerythrin reduced p21 expression in all studied cases, while BIM inhibited the p21 expression in 4 of 5 studied cases (fig. 4B). The use of PKC inhibitors only affected the expression of p21, and not the localization as is demonstrated in fig. 4B. Rb was used as a fractionation control. These results underscore a role for PKC-dependent activation of p21 expression in malignant monocytic cells.

Next, we investigated whether the activated signal transduction pathway leading to constitutive p21 expression might be due to the autocrine production of a growth factor or a cell-autonomous effect. Therefore, after 3 days of culture conditioned medium of monocytic AML blasts (M4-5) cultures (supernatant) was added to cultures of myeloid AML blasts (M1-2), and p21 expression was analysed (fig. 4C). In one case, monocytic AML conditioned medium induced p21 expression in myeloid AML blasts, while in the additional cases no effects were observed. Since these myeloid AML cells might have some inherent blockade, the conditioned medium was also added to monocytes of healthy donors. The AML conditioned medium now induced p21 expression in normal monocytes, although not as high as PMA or GM-CSF induced p21 expression (fig. 4C). These results suggest that in monocytic leukemia the autocrine production of growth factors could lead to constitutive p21 expression. In addition, a cell-autonomous mechanism of p21 expression can not be excluded and might also contribute to this observation.

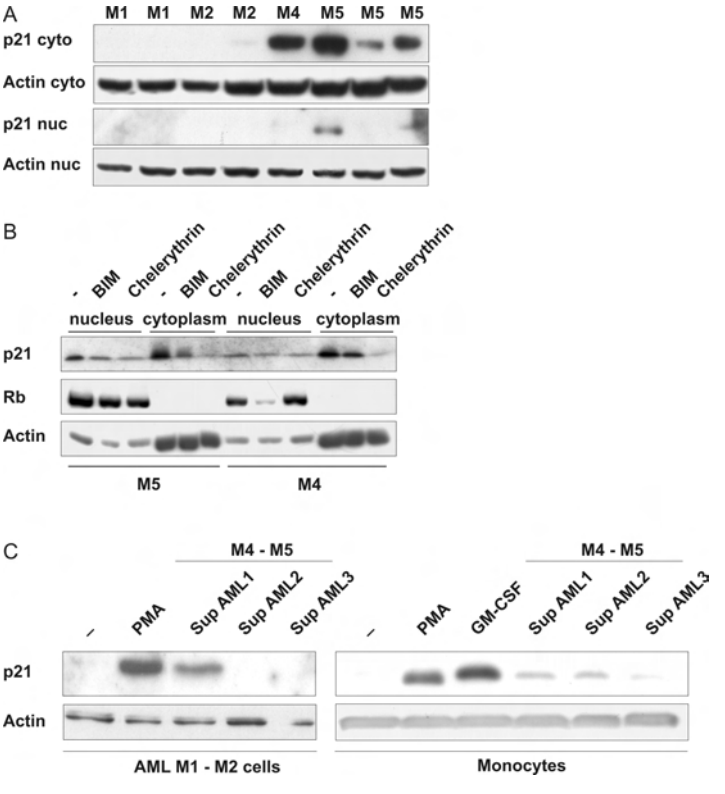

Figure 4 Expression of p21 in AML blasts.
(A) AML blasts were isolated and cultured as described. Cytoplasmic and nuclear fractions were analysed for p21 content by SDS-PAGE. 8 representative cases are shown. The data presented is representative for 2 individual experiments. (B) AML blasts (M4 -M5) were isolated and cultured for 24 hrs in the presence of 1 µM BIM or 6 µM Chelerythrin. The p21 protein was detected in cytoplasmic fractions by means of SDS-PAGE. As controls for sub-cellular fractionation Rb protein was used. Two representative cases are shown. The data presented are representative for 2 individual experiments. (C) AML blasts (M1-2) were cultured for 24 hrs and stimulated with 50 nM PMA or conditioned AML medium from M4-5 cultures to study the role of autocrine growth factors leading to p21 expression. Conditioned AML medium was obtained by culturing $3x10^6$ AML blasts (M4 -5) for 3 days. The same conditioned media were added to normal monocytes in order to analyse p21 expression. Stimulation with 50 nM PMA or 10 ng/ml GM-CSF was used as positive control. Actin is shown as loading control. The data presented are representative for 2 individual experiments. Cyto = cytoplasmic, nuc = nuclear.

Cellular function of p21

It has been demonstrated that cytoplasmic p21 has anti-apoptotic properties in response to stress due to the interaction between p21 and ASK1.[182] To investigate whether this applies also to the effects of cytostatic agents, U937 cells overexpressing p21 and neomycin transfected control cells were exposed to VP16 or H_2O_2. A significant lower percentage of apoptotic cells was

observed in response to VP16 exposure in p21-overexpressing cells compared to control cells (20.0% ± 0.9 vs. 55.8% ± 3.8, N=8, p<0.01) (fig. 5A). In addition p21 negative (N=6) and p21 positive (N=7) AML blasts were subjected to VP16 treatment. As a measure of the apoptotic response, levels of activated caspase 3 were detected, as well as combined annexin V/PI stainings, which were both measured using flow cytometry. The group of AML blasts with high cytoplasmic p21 expression (N=7), was significantly less sensitive to VP16-induced apoptosis as compared to AML blasts with low p21 expression levels (N=6), both for the active caspase 3 assay (respectively, 12.3% vs. 42.3%, p<0.01) and the combined annexin V/PI experiments (respectively, 8.5% vs. 25.8%, p<0.01) (fig. 5B). Next it was investigated whether the reduced apoptosis corresponded with a reduced phosphorylation, and hence activation of known downstream targets of ASK1, p38 and JNK, since these have been shown to be effectors of ASK1 mediated apoptosis.[115;322;323] As depicted in fig. 5C, the protective effect of p21 in the U937 cell-line was associated with a reduced phosphorylation of p38 and JNK in p21 overexpressing cells in response to VP-16 triggering. Similar results were obtained with the stress response activator H_2O_2 (fig. 5D). To further underscore the connection between p21 and ASK1, co-immunoprecipitations were performed with p21 and ASK1 in two AML cases with high cytoplasmic p21 levels. For this co-immunoprecipitation, an anti-p21 antibody conjugated to agarose beads was used. As control for p21 expression and p21-ASK1 complex formation, untreated AS-E2 cells stimulated with 50 nM PMA for 16 hours were used. As negative control unstimulated AS-E2 cells were used, since unstimulated AS-E2 cells do not express p21 (fig. 2A). After 16 hours of PMA (50nM) stimulation, complex formation between p21 and ASK1 could be observed in the AS-E2 cell-line, indicating that ASK1 co-precipitates with the p21 protein. (fig. 6). In the AML blasts, ASK1 coprecipitated with p21 in all conditions shown. Although in the M5 AML cells PMA treatment resulted in more ASK1 immunoreactivity, this is probably due to increased p21 expression in response to PMA. The complex formation between p21 and ASK1 itself however, does not appear to be PMA dependent, since ASK1 can also be detected in p21 precipitates from untreated M5 AML cells. In short, we show that in monocytic leukemias cytoplasmic p21 functions as an anti-apoptotic protein. This is demonstrated by a lowered sensitivity towards VP16-induced apoptosis, which is reflected by a decreased phosphorylation of known downstream targets of ASK1, p38 and JNK. The observed complex formation between p21 and ASK1 further strengthens this hypothesis.

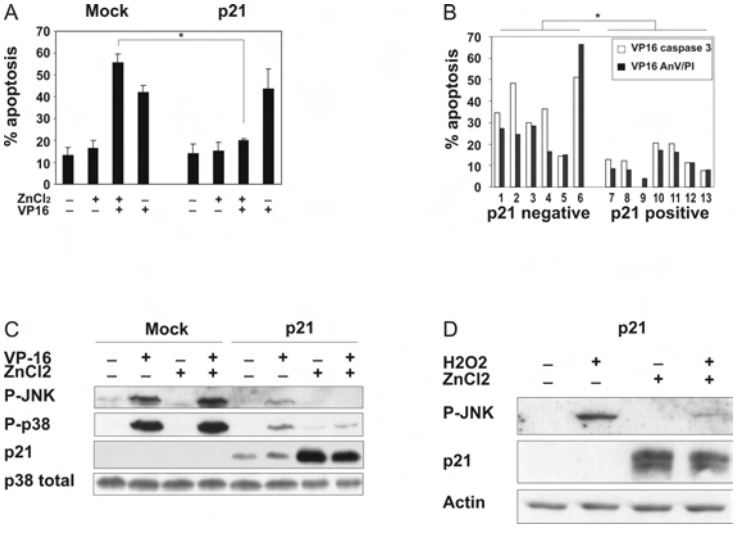

Figure 5 The p21 protein affects apoptosis induced by VP16 or H_2O_2.
(A) Mock transfected U937 cells or U937 cells stable transfected with a $ZnCl_2$ inducible p21 construct were cultured for 3 days in the presence of 180 µM $ZnCl_2$ to induce p21 expression. Next, the cells were exposed to 20 µg/ml VP16 for 24 hrs and afterwards apoptosis was measured using the combined annexin V/PI staining procedure as described in materials and methods. The data presented are averaged from eight independent experiments, with error bars denoting standard errors. * = P<0.01. (B) AML blasts were isolated and cultured as described. After 24 hours of exposure to 20 µg/ml VP16, apoptosis was measured using the combined annexin V/PI staining by flow cytometry. Also levels of activated caspase 3 were measured using flow cytometry as described in material and methods. For both experiments * = P<0.01. (C) The mock and stable transfected U937 cells expressing p21 were cultured for 3 days with 180 µM $ZnCl_2$ and apoptosis was induced by culturing in the presence of 20 µg/ml VP16. Activation of JNK and p38 were studied in total cell extracts by western analysis. The data presented are representative for 5 individual experiments. (D) Apoptosis was induced in U937 cells stable overexpressing p21, by culturing these cells 24 hrs in the presence of 300 µM H_2O_2. Whole extracts were assayed for phosphorylated JNK by western analysis. The data presented are representative for 5 individual experiments.

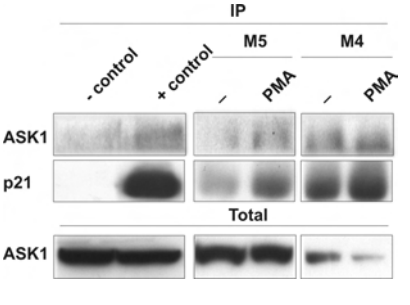

Figure 6 The p21 protein forms a complex with ASK1 in monocytic AML blasts and AS-E2 cells.
10^7 AML blasts (M4 -5) were cultured in the absence or presence of 50 nM PMA for 16 hrs as indicated. Cell lysates were subjected to p21 immunoprecipitation (IP) using an α-p21 antibody, conjugated to agarose beads. Immunoblotting was performed using α-p21 and α-ASK1 antibodies. Expression of ASK1 in all samples was confirmed by immunoblotting of total cell extracts, see the "input" panel. As negative and positive controls, 10^7 AS-E2 cells were grown in the absence or presence of 50nM PMA respectively and IP was performed. The data presented are representative of five individual experiments.

Discussion

The role of nuclear p21 as cell cycle regulator by interaction with several cyclin dependent kinases is well established.[302-306] Recent studies have indicated that p21 might have an additional function when the protein is localized in the cytoplasm. Cytoplasmic p21 is linked to the anti-apoptotic process by interacting with ASK1 or pro-caspase 3.[182;308;324;325] The present study indicates that in differentiating hematopoietic cells of monocytic and myeloid lineage, p21 can selectively be upregulated by a number of cytokines, which in monocytic cells, is a PKC-dependent but NF-κB-independent process. A recent study in monocytes indicated a link between NF-κB activation and p21 upregulation, suggesting that p21 might accomplish the anti-apoptotic effects of NF-κB.[183] However, in the present study it is shown that NF-κB activity is not required for p21 expression. In monocytic leukemia, constitutive expressed p21 is frequently observed in contrast to myeloid leukemia, which is a PKC dependent process. This is likely linked to the autocrine production of growth factors. However, it is not strictly excluded that a yet unknown upstream kinase or receptor mutation results in constitutive activation of this signal transduction pathway leading to constitutive p21 expression. The PKC-dependent expression of p21 is not linked to the MEK1 or PI(3)K signaling pathways. This is in contrast with other reports, where it was shown that p21 protein stability is enhanced by phosphorylation through PKB,[309] and, where PMA-induced p21 expression is linked to nuclear distributed ERK.[326] In addition, phosphorylation of p21 on Thr[145] is suggested to be a prerequisite for its cytoplasmic localization.[327] Whether the localization of p21 is dependent on the phosphorylation status of the protein in conjunction with the cellular setting is the subject of further research.

Finally it is shown that p21 overexpression inhibits VP16-mediated apoptosis as is reflected by the inhibitory effects on the phosphorylation of p38 and JNK, known downstream targets of ASK1. In addition, p21 and ASK1 form a complex in cell-lines and AML blasts, underscoring a strong connection between the anti-apoptotic function of p21 and ASK1-mediated apoptosis. The obtained results in monocytes are in disagreement with findings by Asada and co-workers, who demonstrated that unstimulated monocytes express p21.[182] The cause of this difference is unclear but might be related to the used isolation procedure.

The data presented here and other findings indicate that in leukemic cells anti-apoptotic proteins are regulated by different signaling routes including the RAS/PI(3)K/NF-κB pathway,[64;276;284;291;294] the STAT3 and STAT5 pathways[40;47]

and in monocytic blasts also at the level of p21 expression.[182-184] These findings illustrate that, until a single oncogenic defect is defined which is responsible for the total spectrum of activation signals, combination of inhibitors have to be used for chemotherapeutic interference with the distinct and overlapping activated signalling routes.

Acknowledgements

We would like to thank Dr. Minoru Asada from the department of Biochemistry, The Cancer Institute, Tokyo, for the kind gift of the U937 cell lines, stable transfected with either mock or a $ZnCl_2$ inducible p21 construct.

HSP27 protects AML cells against VP-16-induced apoptotis through modulation of p38 and c-Jun

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Chapter 5

Abstract

Objective. To investigate 1) the signal transduction pathways affected by heat shock protein 27 (HSP27) expression; and 2) the expression and regulation of HSP27 in acute myeloid leukemia (AML).
Materials and methods. RNA interference studies for HSP27 in leukemic TF-1 cells were used to investigate the effects on downstream signal transduction and apoptosis after VP-16 and CD95/Fas treatment. HSP27 expression and activation was investigated in AML blasts through western blot analysis.
Results. RNA interference for HSP27 resulted in a twofold increase in VP-16-induced apoptosis, which was preceded by enhanced p38 and c-Jun phosphorylation and a two-fold increased cytochrome c release into the cytoplasm. DAXX co-immunoprecipitated with HSP27 suggesting an inhibitory role of HSP27 in VP-16-mediated activation of the ASK1/p38/JNK pathway. CD95/Fas-induced apoptosis however was unaffected by HSP27 siRNA, due to upregulation of HSP27. Although HSP27 was highly expressed and phosphorylated in primitive monocytic AML blasts (M4-M5, 91%, N = 11) and undetectable in myeloid blasts (M1-M2, N = 5), VP-16-mediated apoptosis correlated moderately with HSP27 expression. This is likely due to the co-expression of $p21^{Waf1/Cip1}$, which is in the majority of the monocytic AML M4-M5 blasts constitutively localised in the cytoplasm. Overexpression of cytoplasmic p21 inhibited the enhanced p38 phosphorylation after HSP27 RNAi, suggesting a predominant anti-apoptotic role of p21 over HSP27.
Conclusion. 1) HSP27 inhibits VP-16-mediated phosphorylation of p38 and c-Jun, cytochrome c release and subsequent apoptosis; 2) HSP27 is expressed and activated in monocytic AML blasts; 3) Cytoplasmic expression of p21 compensates for the lack of HSP27.

Introduction

Acute myeloid leukemia (AML) is characterized by an accumulation of immature cells in the bone marrow, resulting in the disruption of normal hematopoiesis.[16;17;300] The leukemic population possesses a growth advantage that is in part linked to the constitutive activation of intracellular proteins that trigger the activation of anti-apoptotic proteins.[40;64;290;294] The small heat shock protein 27 (HSP27) is a member of the heat shock protein (Hsp) family, whose expression is transiently induced in response to stress. It has been demonstrated that HSP27 levels change during cellular stress[328] and differentiation,[329-331] both on the transcriptional and posttranslational level.[328] In normal cells, HSP27 mainly exists in large oligomeric units up to 800 kDa in size. Stress leads to changes in the multimeric status of the protein due to phosphorylation of HSP27 on three serine residues,[117] that can change the activity of the protein.[332] HSP27, besides being involved in cytoskeletal stability, cell motility and its function as a chaperone,[328;332-334] has been implicated in apoptosis.[113;114;331;335-337]

Induction of apoptosis can occur via an intrinsic as well as an extrinsic signal transduction pathway. The classical intrinsic pathway is initiated through the release of cytochrome c from mitochondria. Cytochrome c interacts with Apoptosis Protease Activating Factor-1 (Apaf-1), which oligomerises and binds to pro-caspase-9.[117] The formation of this caspase-activating complex, termed the apoptosome, results in the activation of caspase-9, which in turn triggers the proteolytic cleavage of pro-caspase-3, leading to apoptosis.[117]
Binding of ligands to death receptors on the cell surface (e.g., CD95/Fas) not only results in the formation of the death inducing signaling complex (DISC) via the recruitment of the adapter molecule FADD, but also to the recruitment of DAXX to the cytosolic end of the CD95/Fas receptor.[111;112] DAXX then binds Apoptosis Signal Regulating Kinase-1 (ASK1), which in turn activates c-Jun N-terminal Kinase (JNK) and p38, leading to cytochrome c release and activation of caspases.[113-116]

The mode of HSP27 action in apoptosis is still unclear but could exert its effect at the level of mitochondrial stability[336;338] or DAXX signaling.[113;114]
Since HSP27 expression has been observed in multiple malignancies[339;340] including AML[341;342] and HSP27 expression is frequently correlated with unfavorable prognosis,[343] we questioned whether HSP27 plays an important role in the protection of AML blasts against CD95/Fas- or VP-16-induced apoptosis; both have been described to activate the DAXX pathway.[113;114;290]

Here we demonstrate that HSP27 is predominantly expressed and phosphorylated in AML blasts of the monocytic lineage. RNA interference indicates that HSP27 affects the VP-16-induced DAXX pathway through the modulation of JNK and p38[117;290] activation, resulting in the enhanced cytochrome c release and induction of apoptosis. Additionally, our results also indicate that in monocytic leukemia, HSP27 is not the determining anti-apoptotic factor, since cytoplasmic localized p21$^{Waf1/Cip1}$ is able to reverse the enhanced VP-16-induced phosphorylation of p38 after RNAi for HSP27.

Materials and methods

Patient population and isolation of AML cells - Peripheral blood cells or bone marrow cells from 16 adult untreated patients with AML were studied after informed consent. The AML cases were defined according to the classification of the French-American-British (FAB) committee as M0-M6.[18] AML blasts were isolated by density-gradient centrifugation. The cells were cryopreserved in aliquots of 20-30 x 10^6 cells in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS; Bodinco, Alkmaar, the Netherlands), employing a method of controlled freezing and storage in liquid nitrogen. After thawing, T-lymphocytes were depleted by 2-aminoethylisothioronium bromide (AET)-treated sheep red blood cell (SRBC) rosetting. The cell population consisted of more than 98% AML blasts as determined by May-Grünwald-Giemsa staining. Fluorescence-activated cell sorting (FACS) analysis demonstrated <1% CD3 (Becton Dickinson, Sunnyvale, California, USA) positive cells.

Preparation of monocytes, granulocytes and CD34$^+$ BM cells - Peripheral blood cells were obtained from healthy volunteer blood donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque density-gradient centrifugation. T- lymphocytes were depleted by AET-treated SRBC rosetting. Monocytes were further enriched by plastic adherence (1h, 37°C, 5% CO_2) and demonstrated a purity >95% detected by FACS analysis with anti-CD14 antibody (Becton Dickinson, Sunnyvale, CA).

Peripheral blood from healthy volunteers, anti-coagulated with 0.32% sodium citrate, was used to isolate granulocytes as described by Fuhler et al (and references therein).[344]

CD34$^+$ cells were isolated from bone marrow from healthy donors by first making mononuclear cell suspensions followed by incubation of the suspension

with phycoerythrin-labelled anti-$CD34^+$ antibody for 30 minutes at 4°C and subsequent FACS sorting using the MoFlo (DakoCytomation, Carpinteria, Ca, USA). $CD34^+/CD36^-$ and $CD34^-/CD36^+$ cell populations were obtained by incubating the AML blasts with phycoerythrin-labelled anti- $CD34^+$ and FITC-labelled anti- $CD36^+$ antibodies for 30 minutes at 4°C. Subsequently, the different cell populations were isolated by FACS sorting using the MoFlo.

Cell culture, viral vectors and transfections - AML blasts were cultured at 37°C, 5% CO_2 at a density of $1x10^6$/ml in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (ICN Biomedicals, Aurora, Ohio, USA) and 10% FBS. Isolated $CD34^+$ cells were cultured in IMDM medium (ICN Biomedicals, Aurora, Ohio, USA) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin. Monocytes were cultured at 37°C, 5% CO_2 at density of 1×10^6 /ml in RPMI 1640 and 10% FBS. The human cell lines U937 (ATCC, Product No. CRL-1593.2), THP-1 (ATCC, Product No. TIB-202) and HL-60 (ATCC, Product No. CCL-240) were cultured in RPMI 1640 supplemented with 10% FBS. The human cell line TF-1 (ATCC, Product No. CRL-2003) was cultured in RPMI 1640 supplemented with 10% FBS and 10 ng/ml GM-CSF (Genetics Institute Cambridge, MA).

pMSCV-p21dNLS was constructed by removing the C-terminal bipartite Nuclear Localization Signal (NLS; RKRR) via PCR from pCMV-p21 (kindly provided by Prof. Dr R.H. Medema and Dr. P. Coffer) and ligating the XhoI-EcoRI fragment into pMSCV-iGFP (kindly provided by Dr. J.J. Schuringa). TF-1 cells were transduced with viral particles collected from 293T cultures transfected with pCL-AMPHO and pMSCV-p21dNLS using FuGENE6 (Roche, Almere, The Netherlands). GFP positive TF-1 cells were isolated by FACS sorting using the MoFlo.

Reagents and antibodies - An antibody against phosphorylated p38 was obtained from New England Biolabs (Beverly, MA, USA). Antibody against HSP27 was purchased from Stressgen (Victoria BC, Canada), anti-actin (C4) was obtained from ICN Biomedicals (Aurora, Ohio, USA). Antibodies against c-Jun, cytochrome c and GAPDH were obtained from Santa Cruz biotechnology (Santa Cruz, Ca, USA), pan Serine from Zymed Laboratories (San Francisco, Ca, USA). Anti-FLAG (M2) was obtained from Sigma. Antibody against p21 was bought from Transduction Laboratories (Lexington, KY, USA). Anti CD34-HPCA2-PE was obtained from Becton Dickinson. And CD36 FITC was obtained from IQ products (Groningen, The Netherlands). Recombinant human (Rh) interleukin (IL)-1ß was obtained from Mekesson HBOC Bioservices (Rockville, MD, USA). Rh Granulocyte Monocyte-Colony Stimulating Factor

(GM-CSF) were purchased from Genetics Institute (Cambridge, MA, USA). The VP-16 was obtained from TEVA (Haarlem, The Netherlands) and the apoptosis inducing monoclonal antibody CD95/Fas (7C11) was obtained from Immunotech (Marseille, France).

Preparation of protein extracts and Western blotting - The amount of HSP27, GAPDH, $p21^{Waf1/Cip1}$ and actin and the degree of phosphorylated p38, c-Jun and pan Serine were determined by SDS-PAGE analysis (sodium dodecyl sulphate-poly acryl amide gel electrophoresis) on whole cell extracts. Cells were harvested and total cell extracts were prepared by resuspending the cells in lysisbuffer (20 mM TrisHCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 µM pepstatin and 1 mM DTT) and kept for 15 min on ice. Binding of each antibody was detected by HRP labeled secondary antibodies using enhanced chemiluminesence (ECL) according to the manufacturer's recommendations (Amersham Life Sciences, Buckinghamshire, UK).

For cytoplasmic and nuclear extracts, cells were harvested and were prepared according to the "mini extracts" method [273]. The extracts were normalized for protein content prior to SDS-PAGE. Proper fractionation and lack of leakage of nuclear proteins to the cytosol was determined by western blotting for the nuclear protein Retinoblastoma (Rb).

RNA interference - Short interfering RNA duplexes for HSP27 were made using the Silencer siRNA construction kit from Ambion (Austin, Texas, USA) according to the manufacturer's protocol. The HSP27 target sequence used is: AAGCTGCAAAATCCGATGAGA. GAPDH control siRNA duplexes are provided within the kit. $2x10^6$ TF-1 cells were transfected with a final concentration of 25 nM of RNAi duplexes using oligofectamine according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). Lysates were prepared at the indicated time points in lysisbuffer and equal amounts of protein were subjected to western blot analysis.

Combined annexin V/PI staining procedure - Viability was assessed using an annexin V staining kit (IQ Products, Groningen, The Netherlands) according to the manufacturer's recommendations. Briefly, after 6 hrs of culture in RPMI 1640 medium supplemented with 10% FBS, with or without addition of VP-16 (20 µg/ml) or CD95/Fas (2 µg/ml), cells were harvested, resuspended in 100 µl calciumbuffer containing 5 µl of annexin V and incubated for 20 min at 4°C in the dark. Cells were washed with 5 ml calciumbuffer and subsequently

incubated in 300 µl calciumbuffer containing 2.5 µl of propidium iodide (PI) for 10 min in the dark at 4°C. Finally, binding of fluorescein-conjugated annexin V and PI was measured by fluorescence-activated cell sorting.

Immunoprecipitation - 10^7 AML blasts were cultured for 16 hrs in RPMI 1640 medium supplemented with 10% FBS. Cells were harvested, washed with ice-cold PBS containing 1 mM sodium orthovanadate and subsequently lysed in 500µl lysisbuffer (20 mM TrisHCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 µM pepstatin and 1 mM DTT) for 15 min on ice. Cell lysates were clarified at 10 000g for 20 min and incubated with 5 µl HSP27 antibody. After 4 hours rotating, protein A sepharose beads were added and incubated O/N at 4°C. Immunocomplexes were washed 3 times with lysisbuffer and separated by SDS-PAGE. Binding of anti-pan-Serine antibody was detected using ECL (Amersham, UK).

$20x10^6$ TF-1 cells were electroporated with 20 µg of pCDNA3 MYC-FLAG-DAXX and 20 µg of pCDNA3 HA-ASK1 at 240V and 960 µF. After 24 hours cells were harvested, and co-immunoprecipitations were performed as described above. Binding of anti-FLAG antibody was detected using ECL.

Cytochrome C ELISA - The Quantikine Human Cytochrome c immunoassay from R&D Systems (Minneapolis, MN, USA) was used to measure cytochrome c levels in the cytoplasm in response to VP-16 or CD95/Fas treatment, according to the manufacturer's recommendations. Cytoplasmic and mitochondrial fractions were separated using the mitochondria isolation kit from Sigma (Zwijndrecht, The Netherlands).

Statistical analysis - The student t-test for paired samples was used to determine statistical significance of the data about apoptosis induction in the TF-1 cell-line. The 2 x 2 contingency test was used to determine association between FAB classification and HSP27 expression.

Results

HSP27 expression in hematopoietic cells

To determine whether HSP27 is expressed in different hematopoietic cells, we isolated granulocytes, monocytes, mononuclear bone marrow cells and $CD34^+$ cells from healthy donors. Mononuclear cells from different donors express HSP27 at equal levels, as well as monocytic cells and $CD34^+$ cells (fig. 1A). In

terminally differentiated granulocytes however, HSP27 could not be detected. A differentiation-dependent expression was also observed in cell lines. HSP27 was highly expressed in the monocytic cell lines THP-1 and U937; moderate expression was observed in the erythroleukemic cell line TF-1 and in the myeloid cell line HL-60, HSP27 could not be detected in (fig. 1B).

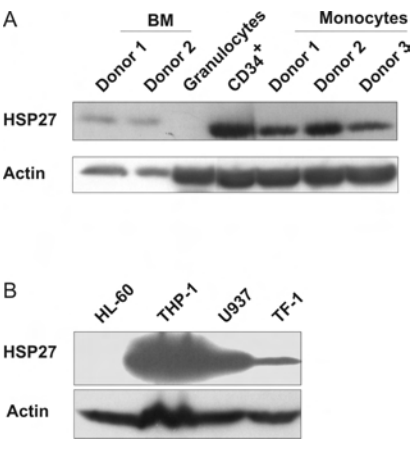

Figure 1 Expression of HSP27 in normal hematopoietic cells and cell lines.
(A) Bone marrow mono nuclear cells, granulocytes and monocytic cells from healthy donors were isolated and cultured as described in material and methods. $CD34^+$ cells were isolated from bone marrow from healthy donors by making mononuclear cell suspensions followed by incubation of the suspension with phycoerythrin-labelled anti-$CD34^+$ antibody for 30 minutes at 4°C and subsequent FACS sorting using the MoFlo. HSP27 expression was investigated by western blot analysis. Actin is shown as loading control. A representative experiment is shown. (B) HL-60, THP-1, U937 and TF-1 cells were cultured as described in the material and methods section, and lysates were analyzed for HSP27 expression using western blot analysis. Actin is shown as loading control. A representative experiment is shown.

RNA interference for HSP27 enhances apoptosis after VP-16 treatment

To investigate whether HSP27 plays a role in the apoptotic process in leukemic cells, RNA interference was used to knock down HSP27 protein levels in TF-1 cells. Figure 2A shows the decrease in HSP27 levels after transfection with short interfering RNAs (siRNAs) in time. After two days HSP27 levels were reduced with approximately 50% compared to the expression levels in untransfected cells (-). A further reduction in HSP27 protein levels was observed three, four and five days after RNAi transfection. The siRNAs used were specific, since no effect on GAPDH expression was observed using HSP27 siRNAs and vice versa (fig. 2B).

To investigate whether HSP27 indeed protects against apoptosis, TF-1 cells were transfected with either HSP27 or GAPDH siRNAs and the percentage of apoptotic cells was determined by means of annexinV/PI staining followed by flow cytometry, 6 hours after incubation with VP-16 or CD95/Fas. VP-16-mediated apoptosis was significantly increased in HSP27-RNAi treated cells compared with the control GAPDH-RNAi treated cells (26.8% ± 8.7 vs. 5.8% ± 3.8, N=5, $p<0.005$, fig. 2C). HSP27 does not seem to be involved in CD95/Fas-induced apoptosis, since knock down of HSP27 did not significantly affect the percentage of apoptotic cells upon treatment with CD95/Fas.

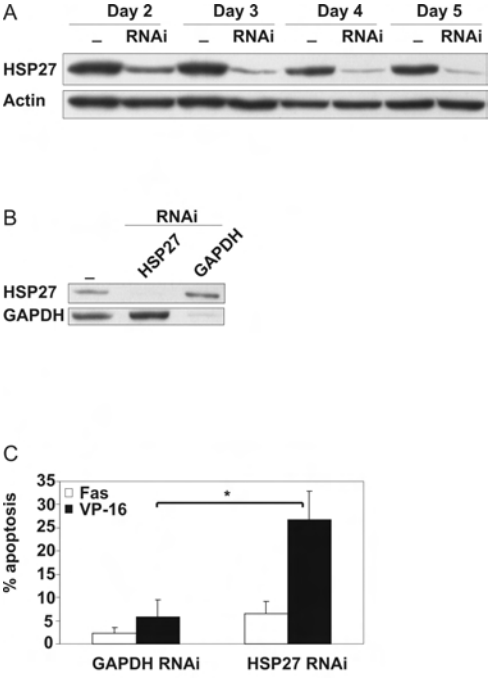

Figure 2 RNA interference for HSP27 specifically knocks down HSP27 expression and enhances VP-16-induced apoptosis.
(A) 2x10^6 TF-1 cells were transfected using oligofectamine with RNA duplexes targeting HSP27, as described in material and methods. On the indicated time points, lysates were made and subjected to western blot analysis for HSP27 expression. Actin is shown as loading control. A representative example of 5 experiments is shown. (B) To indicate specificity, TF-1 cells were also transfected with RNA duplexes targeting GAPDH. Western blot analysis on day 4 indicates that both RNA duplexes are specific. A representative example of 5 experiments is shown. (C) 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 cells were treated with VP-16 (20 μg/ml) or CD95/Fas (2 μg/ml) for 6 hours. Apoptosis was measured with Annexin V/PI staining using flow cytometry as described in material and methods. A representative example of 5 independent experiments performed in triplicate is shown. (* = p< 0.005).

RNA interference for HSP27 enhances cytochrome c release from the mitochondria in response to VP-16

Next we investigated at which level HSP27 affected the VP-16-induced apoptotic response. HSP27 has been described to interact with pro-caspase 3,[338] and cytochrome c,[336] ultimately preventing the activation of pro-caspase 3 by caspase 9-mediated proteolysis. Co-immunoprecipitation studies could not detect complex formation between HSP27 and either pro-caspase 3 or cytochrome c in TF-1 cells, which corresponds with observed cleavage of pro-caspase-3 after CD95/Fas treatment (data not shown).

Next we investigated whether HSP27-blocked cytochrome c release from mitochondria.[345] To investigate whether HSP27 inhibits the release of cytochrome c upon VP-16 and CD95/Fas treatment, we performed ELISAs (Enzyme Linked Immuno Sorbent Assays) on cytosolic extracts from VP-16 and CD95/Fas treated cells. Control GAPDH siRNA treated cells showed a 2-fold increase in cytocrome c release upon VP-16 treatment (fig. 3A) and a 1.5-fold increase was found after CD95/Fas treatment. Knockdown of HSP27 resulted in a 4-fold increase in cytochrome c release upon VP-16 treatment, whereas the effect of CD95/Fas was not altered (fig. 3A). These results

indicate that HSP27 interferes with VP-16-induced cytochrome c release from mitochondria.

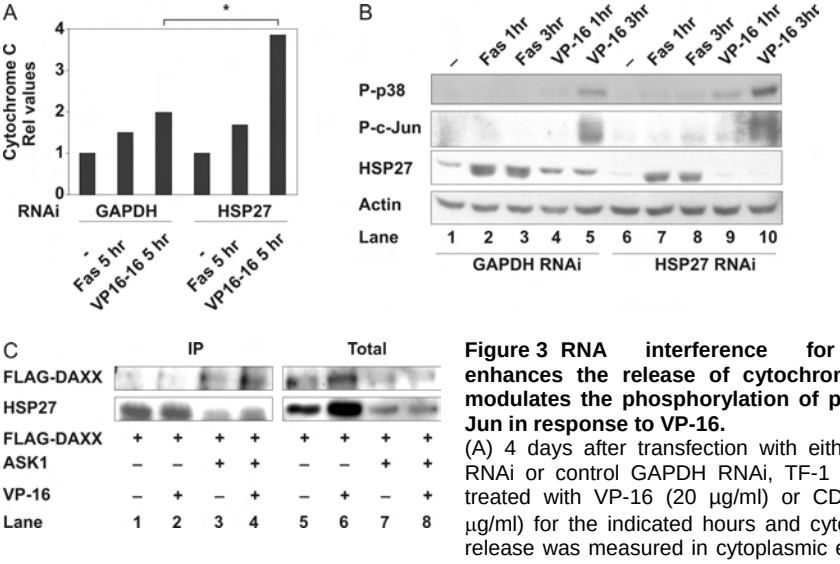

Figure 3 RNA interference for HSP27 enhances the release of cytochrome c and modulates the phosphorylation of p38 and c-Jun in response to VP-16.
(A) 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 cells were treated with VP-16 (20 μg/ml) or CD95/Fas (2 μg/ml) for the indicated hours and cytochrome c release was measured in cytoplasmic extracts by ELISA, as described in material and methods. Relative values, normalized against untreated cells are shown. A representative case of three individual experiments is shown. (B) 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 cells were treated with VP-16 (20 μg/ml) or CD95/Fas (2 μg/ml) for 1 or 3 hours and subjected to western blot analysis for phosphorylated p38, c-Jun and HSP27. Actin is shown as loading control. A representative example is shown. (C) 20 x10^6 TF-1 cells were electroporated with 20 μg of pCDNA3 MYC-FLAG-DAXX with or without 20 μg of pCDNA3 HA-ASK1. After 24 hours cells were lysed in 500 μl lysisbuffer. By immunoprecipitation (IP) of HSP27 and western blot analysis for anti-FLAG, complex formation between HSP27 and DAXX was investigated. Immunocomplexes were detected using ECL. A representative example is shown.

RNA interference for HSP27 influences phosphorylation of p38 and c-Jun after VP-16 treatment

Cytochrome c release from mitochondria has been shown to be involved in JNK-, c-Jun-mediated cell death.[116;346] Therefore we focused on the downstream effectors of VP-16- and CD95/Fas-induced apoptosis, i.e. the stress-activated protein kinase (SAPK, also known as JNK; c-Jun amino-terminal kinase) and the p38 MAP kinase.[115] TF-1 cells transfected with either HSP27 or GAPDH siRNAs were treated with VP-16 or CD95/Fas for one and three hours. P38 and c-Jun activation were determined using western blot analysis (fig. 3B). VP-16 induced a strong activation of p38 and c-Jun in the GAPDH RNAi transfected TF-1 cells after three hours, whereas no activation was observed using CD95/Fas (lanes 2 and 3). RNA interference for HSP27 further increased VP-16-induced p38 phosphorylation levels approximately 3-fold (compare fig. 3B lanes 5 and 10). In addition, the kinetics of p38

phosphorylation were also changed, since phosphorylation of p38 was already observed after 1 hour of VP-16 treatment in HSP27 RNAi treated cells (compare lanes 4 and 9). A similar effect was found on c-Jun phosphorylation after three hours of VP-16 treatment.

In accordance with the absence of enhanced apoptosis, CD95/Fas had no effect on p38 and c-Jun phosphorylation, although JNK has been reported to be a downstream target of CD95/Fas-induced apoptosis by caspase-independent pathways.[113] This coincided with an upregulation of HSP27 expression after CD95/Fas treatment, that was not only observed in the control cells, but also in the HSP27 RNAi transfected cells, despite the presence of the siRNA (fig. 3B, compare lanes 1, 2 and 3 to 6, 7 and 8 respectively). In non-transfected HL-60 cells CD95/Fas treatment also induced HSP27 expression (data not shown). In conclusion, these data demonstrate that knock down of HSP27 leads to an enhanced activation of p38 en c-Jun in response to VP-16, indicating a role for HSP27 upstream from mitochondria.

HSP27 forms a complex with DAXX in an is ASK-1 dependent manner

Since HSP27 has been described to interact with DAXX,[113;114] which is located upstream of ASK1, an activator of p38 and JNK,[323;347] we performed co-immunoprecipitation studies to investigate whether HSP27 interacts with DAXX. TF1 cells were transfected with FLAG-DAXX and ASK1 expression plasmids as indicated. In figure 3C, we show that complex formation between DAXX and HSP27 occurs and that this is ASK1 dependent. (fig. 3B, panel IP). In the absence of ASK1, FLAG-DAXX could not be detected in the precipitates (lanes 1 and 2). Co-expression of both ASK1 and DAXX led to massive cell death (data not shown), resulting in lower amounts of protein in the ASK1/DAXX transfected samples (totals, lanes 3 and 4).

Summarizing, these data indicate complex formation between HSP27 and DAXX in TF-1 cells and suggest a protective effect of HSP27 against VP-16-induced DAXX/JNK/p38 activation.

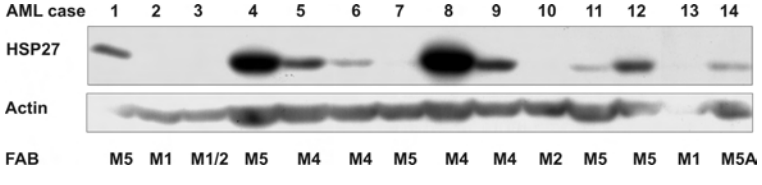

Figure 4 HSP27 is differently expressed in AML samples.
Acute myeloid leukemic cells were isolated and cultured as described in material and methods and HSP27 expression was investigated using western blot analysis. A representative case of 3 experiments is shown. The 2 X 2 contingency test was used to determine association between FAB classification and HSP27 expression (correlation, $p<0.005$).

HSP27 expression in Acute Myeloid Leukemia

In the erythroleukemic cell line TF-1 we demonstrated that knockdown of HSP27 enhances VP-16 mediated apoptosis via modulation of p38 and c-Jun phosphorylation and subsequent mitochondrial cytochrome c release, which eventually results in enhanced apoptosis.

Therefore we questioned whether HSP27 also protects AML blasts against an apoptotic insult. In view of the high expression of HSP27 in $CD34^+$ and monocytic cells (fig. 1A), we first studied HSP27 expression levels in myeloid (FAB M1/M2, N = 5) and monocytic (FAB M4/M5, N = 11) AML samples. None of the AML M1/M2 cases expressed HSP27, despite the presence of high CD34 antigen levels in 60% of these cases (fig. 4 and table 1). In contrast, 10 out of 11 AML M4/M5 patients demonstrated distinct, although variable, HSP27 expression (correlation $p<0.005$; fig. 4 and table 1). Within these AML patients, HSP27 is most prominently expressed within immature AML progenitors ($CD34^+/CD36^-$) as compared to the more differentiated AML cells ($CD34^-/CD36^+$), investigated through FACS sorting and western blot analysis (N = 2, data not shown). To exclude that experimental procedures modulated HSP27 levels, fresh AML blast and cryopreserved blasts of the same patient were compared, directly and several hours after isolation. No effect of cryopreservation and isolation procedures were observed on HSP27 levels (data not shown).

Table I. FAB classifications, percentage of apoptotic cells, expression patterns and CD34 percentages in patients with AML.

AML case	FAB	Apoptosis % VP-16	Apoptosis % Fas	Hsp27	p21	% CD34+ cells
1	M5	8	16	++	+++	3
2	M1	n.d. *	n.d. *	-	n.d.	1
3	M1/2	27	14	-	-	40
4	M5	9	2	+++	+++	3
5	M4	17	5	++	+++	nd
6	M4	16	21	+	+++	nd
7	M5	16	42	-	+/-	<1
8	M4	4	28	+++	++	5
9	M4	28	2	++	-	2
10	M2	16	20	-	-	8
11	M5	11	12	+	++	3
12	M5	8	5	++	++	1
13	M1	25	49	-	-	91
14	M5A	51	8	+	-	nd
15	M1	16	1	-	+/-	80
16	M5	14	3	+++	-	64
TF-1		20	10	+	-	

n.d. = not determined, spontaneous apoptosis > 90%, FAB = French-American-British classification, VP-16 = percentage of apoptosis induced by VP-16; Fas = percentage of apoptosis induced by CD95/Fas, HSP27 = HSP27 expression levels; p21 = $p21^{waf1/Cip1}$ expression levels, % CD34+ cells = the percentage of CD34 positive cells as determined by FACS analysis.

Expression of HSP27 is not correlated with decreased apoptosis in Acute Monocytic Leukemia

In view of the expression of HSP27 in acute monocytic leukemia we questioned whether HSP27 expression was related to sensitivity towards apoptotic stimuli. Both myeloid (FAB M1/M2) and monocytic (FAB M4/M5) AML blasts were incubated with VP-16 or CD95/Fas. After 6 hours of incubation, the percentage of apoptotic cells was determined by Annexin V/PI staining. In the group without HSP27 expression more than 15% VP-16-mediated apoptosis was found in all cases studied, whereas 60% of the HSP27 expressing cases demonstrated less than 15% apoptosis. However, when all samples were included, no absolute correlation was observed between VP-16- and CD95/Fas-induced apoptosis and HSP27 expression levels. (fig. 5A and table 1).

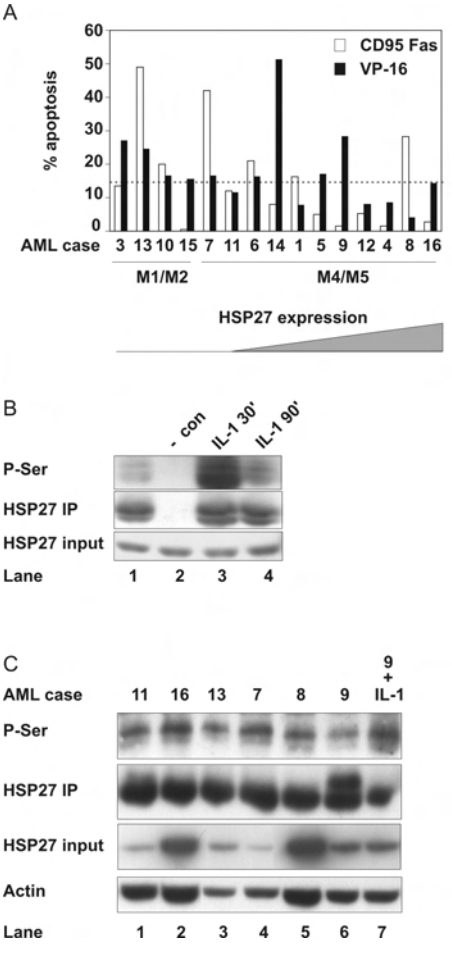

Figure 5 VP-16 and CD95/Fas mediated apoptosis and HSP27 phosphorylation in AML cells.
(A) Apoptosis was induced by culturing the AML cells in the presence of VP-16 (20 μg/ml) or CD95/Fas (2 μg/ml). After 6 hours of culture, binding of fluorescein-conjugated annexin V and PI was measured by fluorescence-activated cell sorting. The Mann-Whitney U test was used to analyse differences in apoptosis between the two groups of differentially expressing HSP27 AML blasts (presence or absence of HSP27 expression and M1/M2 versus M4/M5 FAB classification), but no significant differences could be observed. The dotted line represents the 15% apoptosis level. A representative example of two experiments is shown. (B) By immunoprecipitation (IP) of HSP27, and western blot analysis for pan-serine phosphorylation, the activation of HSP27 was investigated. As control for the HSP27 IP and phosphorylation analysis, performed in the AML cases, the procedure was tested in TF-1 cells. The first lane shows untreated TF-1 cells. Lane 2 shows the IP procedure without antibody, indicating specific pull down of HSP27 in the other lanes. Lane 3 shows TF-1 cells treated for 30 minutes with 10 ng/ml IL-1ß and lane 4 shows 90 minutes of treatment with 10 ng/ml IL-1ß, indicating the transient phosphorylation in TF-1 cells. The HSP27 input is shown here as loading control and this is the HSP27 in total cell lysates before the IP procedure. A representative example is shown. (C) 107 AML blasts were cultured for 16 hrs in RPMI 1640 medium supplemented with 10% FBS. Cell lysis and IP of HSP27 is performed as described in material and methods. The activation of HSP27 was investigated as

described above. Input indicates HSP27 levels in total lysates before IP. 6 representative cases are shown. In order to show that indeed phospho HSP27 is detected, AML case 9 is also treated with 10 ng/ml IL-1ß, a known stimulator of HSP26 phosphorylation. Actin is shown as loading control.

Phosphorylation and activation of HSP27

Since no distinct correlation between HSP27 expression and decreased apoptosis were detected, we wondered whether HSP27 is phosphorylated and activated in AML cells. To investigate whether the HSP27 protein detected in the monocytic AML samples is phosphorylated, immunoprecipitations (IP) were performed on AML lysates using an anti-HSP27 antibody. Following IP, western blot analysis was performed with an anti-phosphoserine antibody (fig. 5B and 5C). Figure 5B shows faint phosphorylation of HSP27 in untreated TF-1 cells (lane 1). In the absence of HSP27 antibodies, HSP27 was not precipitated (lane 2). IL-1 treatment, a known activator of HSP27, resulted in a transient increase in HSP27 serine phosphorylation (lanes 3 and 4).[117;348;349] The finding of phosphorylated and hence activated HSP27 in the TF-1 cell line is in accordance with the finding that it interacts with DAXX in these cells, since this phosphorylated dimer of HSP27 has been described to interact with DAXX.[113] Finally, figure 5C depicts the results of 6 AML cases. Case nine shows IL-1 stimulated AML blasts as a positive control. These results demonstrate that HSP27 is phosphorylated in all AML cases studied irrespective of apoptotic sensitivity.

The cyclin dependent kinase inhibitor $p21^{Waf1/Cip1}$ is co-expressed with HSP27 in monocytic AML blasts and can block the enhanced VP-16-induced apoptosis after HSP27 RNAi

The variability in the results of the sensitivity of monocytic leukemic cells towards apoptotic stimuli might be related to other (more dominant) factors that affect the apoptotic process. As recently described and confirmed in this study (table 1, fig. 6A, similar to fig 4B, with western blot for p21), monocytic leukemic cells frequently express the cyclin dependent kinase inhibitor $p21^{Waf1/Cip1}$ in the cytoplasm, which counteracts the effects of VP-16 on the apoptotic program.[290] In 70 % of the HSP27 expressing AML cases, p21 was co-expressed, whereas in the AML cases with no HSP27 expression, p21 was expressed in 40% of the cases, indicating that at least two proteins are protective in many monocytic leukemia's. We therefore investigated whether p21 could inhibit the enhanced effect on VP-16-induced apoptosis after HSP27 RNAi by using western blot analysis for phosphorylated p38. TF-1 cells were virally transduced with an expression construct for cytoplasmic p21 (p21dNLS). This p21dNLS lacks the bipartite nuclear localization signal (NLS) and is therefore retained in the cytoplasm, where it has been shown to inhibit apoptosis.[182;184;290] Figure 6B

demonstrates cytoplasmic localization of the p21 dNLS protein in TF-1 cells. These cells were transfected with HSP27 or GAPDH siRNAs and treated with VP-16 for 3 h. Cell lysates were prepared and subjected to western blot analysis for the activation of p38. Figure 6C demonstrates that HSP27 siRNAs reduced HSP27 levels in both mock and p21 infected TF-1 cells and that p38 phosphorylation is only increased in response to VP-16 treatment in mock transfected HSP27 siRNA TF-1 cells. (Compare lanes 2 and 4 with 6 and 8). These data suggest that cytoplasmic p21 can compensate for a lack of HSP27 and block VP-16-induced apoptosis via the p38 pathway.

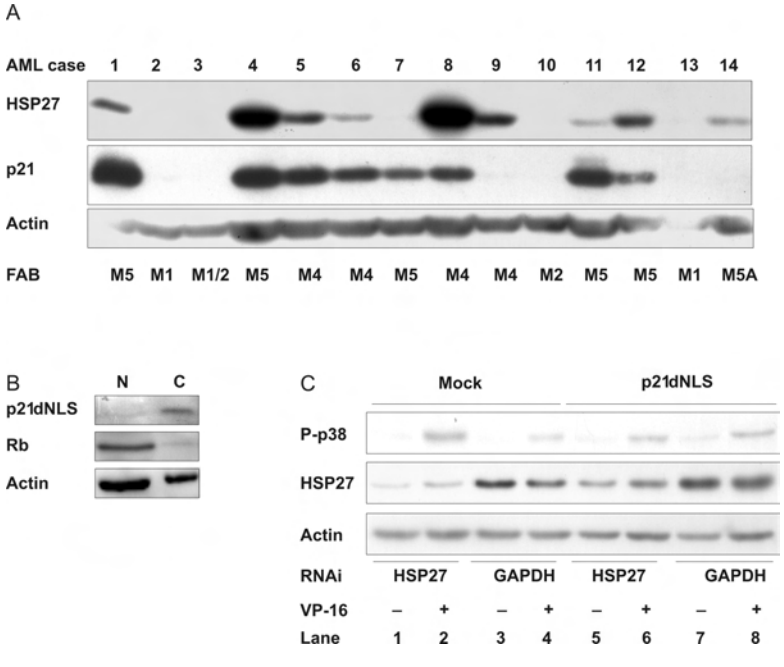

Figure 6 p21 is co-expressed with HSP27 and cytoplasmic p21 (p21dNLS) inhibits enhanced phosphorylation of p38 after RNA interference for HSP27 in response to VP-16. (A) Acute myeloid leukemic cells were isolated and cultured as described in material and methods. HSP27 (same as fig. 4A) and p21 expression was investigated using western blot analysis. (B) TF-1 cells were virally transduced with pMSCV-p21dNLS and nuclear and cytoplasmic fractions were isolated as described in material and methods. Western blot analysis was performed on p21 to demonstrate cytoplasmic localization. Rb is shown to indicate proper fractionation. Actin is shown as loading control. (C) 4 days after transfection with either HSP27 RNAi or control GAPDH RNAi, TF-1 Mock or TF-1 p21dNLS cells were treated with VP-16 (20 µg/ml) for 3 hours and subjected to western blot analysis for phosphorylated p38 and HSP27. Actin is shown as loading control. A representative example is shown.

Discussion

In this study we investigated the potential mechanisms by which HSP27 modulates the apoptotic process in AML blast cells, since co-expression of HSP27 with additional proteins is linked to an unfavorable prognosis.[341;342] Our results demonstrate lineage-restricted expression of HSP27 in normal hematopoietic cells and their malignant counterparts. Normal $CD34^+$ cells and monocytes express high levels of HSP27, whereas in AML cells, HSP27 expression is predominantly restricted to monocytic leukemia. HSP27 appeared to be more expressed in more primitive leukemic progenitor cells, i.e. the $CD34^+/CD36^-$ sorted cell fraction, as has been demonstrated for other anti-apoptotic proteins,[350] underscoring a functional relevance of HSP27 in the malignant stem cell compartment.

However, functional studies with VP-16 did not show a uniform response pattern in monocytic leukemia with regard to VP-16-mediated apoptosis, although HSP27 was phosphorylated in all AML cases. An explanation for this apparent discrepancy is the expression of additional anti-apoptotic proteins including $p21^{Waf1/Cip1}$, which is cytoplasmic localized in monocytic leukemia and interferes with the VP-16-mediated p38 and c-Jun phosphorylation.[290] Overexpression of a cytoplasmic form of p21 in the TF-1 cell-line reversed the VP-16-induced effect seen on the activation of p38 after HSP27 RNAi, indicating that p21 can substitute for HSP27 as an anti-apoptotic factor. Functional studies demonstrate that HSP27 is an important anti-apoptotic factor in the VP-16-induced apoptotic process. Both VP-16 and CD95/Fas can induce apoptosis through the extrinsic signal transduction pathway .[117;290] Induction of apoptosis through this pathway involves translocation of the nuclear protein DAXX to the membrane, where it can bind to the cytosolic end of the CD95/Fas receptor.[351] Following binding to the CD95/Fas receptor, DAXX binds to ASK1.[117] ASK1 activation in turn leads to activation of the stress-activated protein kinase (SAPK, also known as JNK; c-Jun amino-terminal kinase) and p38 subgroups of MAP kinases,[115;347] which have also been described to occur upon VP-16 treatment.[290]

The results presented here indicate that HSP27 RNA interference resulted in enhanced phosphorylation of p38 and c-Jun (a target of JNK) in response to VP-16, but not to CD95/Fas stimulation. In addition, HSP27 RNA interference resulted in an increased release of cytochrome c to the cytoplasm, which has been shown to require ASK1/JNK activation.[116;346;352] The kinetics of these results suggest that cytochrome c release is downstream of the DAXX/ASK1/JNK pathway. c-Jun and p38 phosphorylation are observed as

early as 1 to 3 hours after VP-16 treatment, followed at 5 hours by a release of cytochrome c to the cytoplasm. The sequential activation rather than parallel activation is confirmed by experimental evidence that ASK1-mediated apoptosis can be blocked by the mitochondrial pore opening inhibitor, cyclosporin A, whereas activation of p38 MAPK/JNK is left unaltered.[353]
Complex formation between HSP27 and DAXX, as confirmed here, further underscores a role for HSP27 at the level of DAXX.[113;114] Since HSP27-DAXX complex formation is ASK1-dependent, it is likely that HSP27 only interacts with DAXX when it acquires a proapoptotic function. This is consistent with a role for HSP27 in blocking DAXX translocation to the cytoplasm,[113] where it exerts a proapoptotic function rather then a transcriptional regulatory function.[354]

Bcl-2 family members are known to play a role in mitochondrial integrity and cytochrome c release.[355] DAXX/ ASK1 activation has been shown to inactivate the anti-apoptotic protein Bcl-2[118] and activate pro-apoptotic proteins such as Bax, Bim and Bid[356] (and references therein). HSP27 expression and hence DAXX/ASK1 inactivation might also influence the balance between pro-, versus anti-apoptotic signals at this level. Since HSP27 and Bcl-2 protect against apoptosis with different efficiencies and different pathways,[357] more work needs to be done to clarify the similarities and differences in the affected signal transduction pathways.
A different response pattern was observed with CD95/Fas. HSP27 levels were strongly upregulated by CD95/Fas in the studied cases. This elevated level of HSP27 subsequently prevented the phosphorylation of p38 and c-Jun. Despite the absence of p38 and c-Jun activation, CD95/Fas did induce apoptosis in TF-1 cells to some extend, although not comparable to VP-16-induced levels (table 1).

It is conceivable that CD95/Fas-induced apoptosis in TF-1 cells does not involve the DAXX pathway, but leads to FADD activation and cleavage of pro-caspase-3.[117] It has been demonstrated that overexpression of HSP27 inhibits DAXX-mediated apoptosis, but not CD95/Fas-induced FADD/caspase-dependent apoptosis.[113] Indeed, cleavage of pro-caspase-3 is observed after CD95/Fas treatment of the TF-1 cells, indicating that CD95/Fas is able to induce apoptosis without p38 and JNK activation.

Overall, these experiments indicate that HSP27 protects leukemic cells from VP-16-induced apoptosis through binding to DAXX and modulation of the activation of the p38/JNK pathways, whereas CD95/Fas-induced apoptosis is not affected, either through the upregulation of HSP27 or because of DAXX-independent signaling through a FADD/caspase-dependent pathway. This report underscores that in AML blasts, multiple anti-apoptotic routes are

activated[47;290;294;341;342] and implicate that strategies should be designed in targeting multiple signal transduction pathways in order to treat this highly malignant disorder.

Recently it has been demonstrated that HSP27 confers resistance to the proteasome inhibitor Bortezomib/PS-341[358] and blockade of HSP27 restores sensitivity towards Bortezomib/PS-341. A similar approach might be relevant in the treatment of monocytic leukemia.

Acknowledgements

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Oncogenic Ras blocks TGF-ß-induced cell cycle arrest by degradation of p27 through a Mek/Erk/SKP2-dependent pathway

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Experimental Hematology 2005; 33:747-757

Chapter 6

Abstract

Objective. To examine 1) whether oncogenic Ras affects TGF-β-mediated cell-cycle arrest in hematopoietic cells; and 2) the downstream signal transduction pathway involved in the interference with TGF-β-induced cell-cycle arrest.
Materials and methods. Two leukemic cell lines bearing N-Ras^{L61} mutations; HL-60 and TF-1, and the M1 cell line with wt Ras were investigated for their response to TGF-β. Signal transduction inhibitors, overexpression and RNA interference studies were performed to investigate the involvement of the various proteins.
Results. Although TGF-β signal transduction was not affected, G0-G1 arrest was absent in HL-60 and TF-1 cells due to the absence of p27. Overexpression of p27 restored TGF-β-induced cell-cycle arrest, as well as interfering in Ras-mediated signaling. The farnesyl transferase inhibitor L744832 and the MEK inhibitor U0126 both restored p27 levels and cell-cycle arrest in response to TGF-β. The absence of p27 protein is due to elevated levels of the ubiquitin ligase SKP2, which complexes with and targets p27 for degradation. RNAi for SKP2 and treatment of these cells with the proteasome inhibitor MG132 restored p27 levels, corresponding with decreasing SKP2 levels after interfering in N-Ras signal transduction. P27, phosphorylated at threonine 187, is nuclear localized in N-Ras containing cells. Mutation of this residue to alanine rendered p27 insensitive to degradation.
Conclusion. 1) N-Ras^{L61} transformed cells lack a G0-G1 arrest upon TGF-β treatment due to absence of p27. 2) p27 is degraded through a MapK-, and SKP2-dependent pathway. 3) Overexpression of p27 results in restoration of cell cycle arrest upon TGF-β treatment.

Introduction

Transforming growth factor-β (TGF-β) and Ras-signal transduction have opposite effects on cell proliferation. Where Ras and downstream MEK/Erk (mitogen activated protein kinase / extracellular signal regulated kinase kinase) signaling are induced in response to growth promoting factors,[268;359] cell growth is arrested in response to TGF-β.[158-160] Binding of TGF-β to its receptor leads to the activation of Smad proteins and modulates the transcription of many target genes, including genes that affect cell fate.[158;159]

TGF-β-induced cell cycle arrest is mediated by various Cyclin Dependent Kinase inhibitors (CDKi) including $p15^{Ink4B}$,[164;165] $p21^{Waf1/Cip1}$,[166;167] $p27^{Kip1}$ [168;169] and $p57^{Kip2}$.[170] Furthermore, the expression and activity of additional proteins such as c-Myc, cyclin A, CDK4/6 and the phosphatase cdc25A are suppressed by TGF-β.[171;172] These events eventually lead to decreased activity of the Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes and ultimately result in cell cycle arrest.

One of the key intermediates in TGF-β-induced cell cycle arrest is the CDKi $p27^{Kip1}$ and alterations in protein levels or localization interfere with the inhibitory activity on CDK2 and TGF-β-induced cell cycle arrest.[171;360-362] Regulation of p27 is primarily controlled by phosphorylation and subsequent degradation or proteolysis by two distinct pathways. Phosphorylation of p27 on Threonine residue 187 by the CDK2-Cyclin E complex targets p27 for ubiquitination in the nucleus by the E3 ubiquitin ligase SKP2.[363-369] This ubiquitinated p27 is then degraded by the 26S proteasome.[367;368;370] Phosphorylation of p27 on other residues, such as Serine 10, Threonine 157 and Threonine 198, leads to cytoplasmic translocation where p27 is either degraded or stabilized.[371-377] Cytoplasmic p27 has no inhibitory effect on the cell cycle.

Besides inactivating mutations in the TGF-β receptors or Smad proteins,[158] TGF-β-mediated cell cycle arrest can be blocked by oncogenic Ras. This has been attributed to Map Kinase-dependent phosphorylation of Smad 2/3 and subsequent impaired nuclear translocation, degradation of Smad 4, or mislocalization of p27 to the cytoplasm.[171;178;179]

In Acute Myeloid Leukemia (AML), cytoplasmic mislocalization of p27 is associated with constitutive Akt phosphorylation and unfavorable prognosis.[155] In addition, Akt phosphorylation might depend upon Ras activation.[294] Based on these data it is conceivable that oncogenic Ras, which is frequently mutated in AML, renders hematopoietic cells insensitive to TGF-β treatment through modulation of p27 function, resulting in a disturbed negative feedback control.

In this report we demonstrate that the leukemic cell lines TF-1 and HL-60, bearing oncogenic N-Ras^{L61} mutations, are insensitive to TGF-β with regard to cell cycle arrest. Surprisingly, instead of p27 mislocalization to the cytoplasm, we found that these leukemic cell lines were devoid of p27. A Ras/MEK/Erk/SKP2-dependent mechanism is responsible for the degradation of p27 and overexpression of p27 in these cells restored TGF-β-induced cell cycle arrest.

Materials and methods

Cell culture, (viral) vectors, and transfections - The human and mouse myeloblastic cell lines HL-60 (ATCC, Manassas, VA, USA, Product No. CCL-240) and M1 (ATCC, Product No. TIB-192) were cultured in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (ICN Biomedicals, Costa Mesa, CA, USA) and 10% FBS (Fetal Bovine Serum) (Bodinco, Alkmaar, the Netherlands). The human erythroleukemia cell line TF-1 (ATCC, Product No. CRL-2003) was cultured in RPMI 1640 supplemented with 10% FBS and 10 ng/ml GM-CSF (granulocyte monocyte-colony stimulating factor; Genetics Institute, Cambridge, MA, USA).
pBabe FLAG-27 was constructed by ligating the EcoRI-XhoI fragment of pCDNA3 FLAG-p27 into the pBabe-Ires-GFP puro plasmid. TF-1 cells were transduced with viral particles collected from Phoenix cultures transfected with pCL-AMPHO and pBabe Flag-p27 using FuGENE6 (Roche, Almere, The Netherlands). Cells stable expressing FLAG-p27 were continuously selected with 1 µg/ml puromycine (Sigma, St. Louis, MO, USA).

TF-1 cells were electroporated (960 µF, 240V) with 15 µg of pCMV Tag2B or pCDNA3 FLAG wt p27 or S10A, T157A, S161A, T162A, S178A or T187A mutant p27 in order to study degradation.

HL-60 and TF-1 cells were either mock electroporated (960 µF, 240V) or (1) with 12.5 µg pGAL4-Elk1, 12.5 µg pGAL4-TK-luc and 5 µg pDM2-LacZ for the Elk-Gal4 transactivation assay, (2) with 12.5 µg 7x SBE (smad binding element) Luciferase and 12.5 µg pDM2-LacZ for the TGF-ß reporter assay or (3) with the p27GL-1609 reporter plasmid [378] in order to study p27 transcription. All reporter transfections were collected in 100 µl reporter lysis buffer (Promega Corp, Leiden, the Netherlands) and subjected to luciferase and β-galactosidase assays as previously described.[379]

Reagents and antibodies - The p16, p27(C-19), p57(C20), P-p27 Ser10, Erk (K-23), N-Ras(C20), Rb(C15) and Smad4, antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Smad3, SKP2, p27 and P-p27 Thr187 were purchased from Zymed Laboratories (South San Francisco, CA, USA). P-Erk antibody was obtained from New England Biolabs (Beverly, MA, USA). Flag antibody, p21 antibody and an antibody against Actin (C4) were respectivily obtained from Sigma, Transduction Laboratories (Lexington, KY, USA) and ICN Biomedicals. All antibodies were reactive for both human and mouse isoforms. Horse radish peroxidase (HRP)-labeled secondary antibodies were obtained from DAKO (Glostrup, Denmark). The farnesyl transferase inhibitor L744832 and the MAP kinase/ERK kinase (MEK) 1 inhibitor U0126 were obtained from Biomol (Plymouth Meeting, PA, USA) and Promega Corp. respectivily. TGF-β was purchased from R&D systems (Minneapolis, MN, USA). MG132 was obtained from Calbiochem (Darmstadt, Germany). Nocodazole, Actinomycin D, Cycloheximide, Puromycin, RNase A, Propidium Iodide and Triton X-100 were obtained from Sigma. Sodium citrate-dihydrate was purchased from Merck (Darmstadt, Germany).

Ras binding reaction - TF-1, HL-60 and M1 cells ($5x10^6$) were incubated in 2 ml RPMI 1640 medium supplemented with 0.5% FBS for 24 hours. Cells were lysed in 400 µL lysis buffer and a Ras binding reaction was performed as previously described.[294]

Mutation analysis of N-Ras - Mutation analysis of N-Ras was performed by single-strand conformation polymorphism (SSCP). Genomic DNA was extracted from HL-60 and TF-1 cells and PCR analysis was performed as previously described.[40;294]

Preparation of protein extracts and Western blot analysis - The amount of p27, p21, p16, p57, Erk, SKP2, Ras, FLAG, Smad3, Smad4 and actin and the degree of phosphorylated p27 and Erk were determined by western blotting on whole cell extracts, cytoplasmic or nuclear extracts. Total cell extracts were prepared by resuspending the cells in lysisbuffer (20 mM TrisHCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 µM pepstatin and 1 mM DTT) and kept for 15 min on ice.

For cytoplasmic and nuclear extracts, cells were lysed according to the “mini extracts” method.[273] All extracts were normalized for protein content prior to SDS-PAGE (sodium dodecyl sulphate-poly acryl amide gel electrophoresis).

Proper fractionation and lack of leakage of nuclear proteins to the cytosol was determined with western blotting for the nuclear protein Retinoblastoma (Rb).

Immunoprecipitation - $2x10^7$ cells were cultured for 24 hours in RPMI 1640 medium supplemented with 10% FBS. Cells were washed with ice-cold PBS and subsequently lysed in 500µl lysis buffer (20 mM TrisHCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 µM pepstatin and 1 mM DTT) for 10 min on ice. Cell lysates were clarified at 5 000g for 10 min and after preclearing with 30 µl Protein A Sepharose beads for 1 hour at 4°C, cell lysates were incubated with 5 µL SKP2 or p27 antibody (Zymed) and rotated for 4 hours at 4°C. Protein A sepharose beads (30 µl) were added to each sample and incubated for 16 hrs at 4°C. The immune complex was washed 3 times with lysis buffer. The immune complexes were heated in sample buffer, separated by SDS PAGE, immunoblotted on PVDF membrane (Millipore) and incubated for 16 hrs with either anti-SKP2 or anti-p27 antibody. Immunocomplexes were detected using ECL.

FACS analysis - TF-1, HL-60 and M1 cells were synchronised with 0.33 µM Nocodazole. After 16 hours cells were washed 3 times and plated at $0.25*10^6$/1.5 ml and treated with TGF-ß (2 ng/ml), 0.001% DMSO, U0126 (10 µM) or L744832 (50 µM) as indicated. After 72 hours cells were washed and incubated with Propidium Iodide (PI) solution (0.1 µg/µl RNase A, 0.96 mg/ml Sodium citrate-dihydrate, 0.02 µg/µl PI, 0.1 % Triton X-100) for 20 minutes at room temperature. Binding of Propidium Iodide was measured using FACS (fluorescence-activated cell sorting) (Becton Dickinson, Sunnyvale, California, USA) and cell cycle analysis was performed using ModFit LT.

Quantitative PCR - RNA extraction, preparation of cDNA and Quantitative PCR (Q-PCR) for Smad7, SnoA, SKP2, p15, p18 and p27 were performed as previously described.[290] Primers used: Smad7 For gcctcggacagctcaattcg; Smad7 Rev cgtccacggctgctgcataa; SnoA For cattctcacagatcacctgac; SnoA Rev gtcttacttctctcacaggatg; SKP2 For ccagcaagacttctgaac; SKP2 Rev ggaggcacagacaggaaa; p15 For cgcgaggagaacaagggcat; p15 Rev gcctcccgaaacggttgact; p18 For gattgccaggagactgctac; p18 rev cgtgtgcttcaccaggaact; p27 For gcaaccgacgattcttctac; p27 Rev gtccattccatgaagtcagc; HPRT For tggcgtcgtgattagtgatg; HPRT Rev gatgtaatccagcaggtcag. Primers for SKP2 and the cyclin dependent kinase inhibitors were designed against conserved regions between the human and mouse genes. Primers for Smad7 and SnoA are human specific.

RNA interference - Short interfering RNA duplexes for SKP2 were made using the Silencer siRNA construction kit from Ambion according to the manufacturers protocol (Austin, Texas, USA). The SKP2 target sequences used: AAAGAGGAGCCCGACAGTGAG and AAGTTGCAGAATCTAAGCCTG. GAPDH control siRNA duplexes are provided within the kit. TF-1 cells were transfected with a final concentration of 50 nM or 100 nM of RNAi duplexes using oligofectamine according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). Lysates were made on the indicated time points in lysisbuffer and equal amounts of protein were subjected to western blot analysis.

Statistical analysis - The student's t-test for paired samples was used to determine statistical significance of the cell cycle data.

Results

p27 expression and TGF-ß-induced cell-cycle arrest in cells containing oncogenic Ras

The human myeloid leukemic cell line HL-60 and the erythroleukemic cell line TF-1, which have been reported to contain oncogenic N-Ras mutations,[294;380] were studied with respect to TGF-β-mediated cell cycle arrest. As a negative control, the myeloid cell-line M1 was used, since this cell line contains wt Ras. SSCP analysis confirmed that HL-60 and TF-1 cells are heterozygous for the N-Ras^{L61} mutation (data not shown). To determine whether Ras is activated in these cells, we performed Ras-pull down assays and determined activation of the downstream effector Erk[268] (fig.1A). In HL-60 and TF-1 cells activated Ras and phospho-p42/44 (P-Erk) were observed, both of which were absent in M1 cells, although antibodies were capable of recognizing mouse isoforms (Ras input lane and Erk, data not shown). To test the effect of TGF-β on the cell-cycle, cells were synchronized by nocodazole treatment for 16 hours and subsequently cultured in the absence or presence of TGF-β. FACS analysis demonstrated a minor or no increase in the percentage of cells in G0-G1 phase of the cell-cycle in response to TGF-β (HL-60: 46.3 ± 0.7 vs. 53.1 ± 0.4; TF-1: 42.9 ± 1.2 vs. 42.1 ± 0.4) (fig. 1B). This in contrast to the M1 cell line, which demonstrates an increase of cells in G0-G1 phase from 48.9 ± 0.5 % to 76.4 ± 2.2 % in response to TGF-β.

Next, western blot analysis was performed in order to determine whether the absence of cell cycle arrest in the oncogenic Ras containing cells is related to p27 dysfunction.[171] In M1 cells, p27 was located in the nuclear compartment (data not shown and box fig. 7B). In contrast, no p27 expression was observed in the N-Ras containing HL-60 and TF-1 cells (fig. 1A). To exclude cell-cycle-dependent regulation of p27, presence of p27 was analyzed for all cell cycle phases after synchronisation. As expected, M1 cells show a cell-cycle-dependent regulation of p27, whereas p27 is absent in all cell-cycle phases in the HL-60 and TF-1 cell line (data not shown). These observations suggest that the absence of p27 in cells containing N-Ras mutations might be involved in the unresponsiveness towards TGF-β.

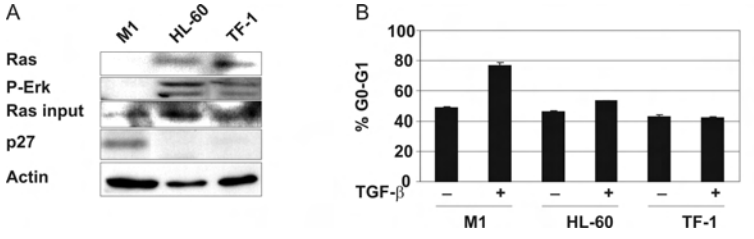

Figure 1 Cells containing N-Ras mutations are unresponsive to TGF-β and are devoid of p27.
(A) TF-1, HL-60 and M1 cell extracts were western analyzed for activated Ras, P-Erk and p27. After synchronisation, cells were harvested and lysed in lysis buffer and a Ras binding reaction was performed as described in material and methods. Ras denotes activated Ras which is precipitated with Raf-GDS glutathione-S-transferase-Ras binding domain (GST-RBD) precoupled to glutathione-sepharose beads. Total lysates show amounts of P-Erk, Ras, Actin and p27 before precipitation. (B) M1, HL-60 and TF-1 cells were synchronized in M-Phase with Nocodazole and subsequently cultured with or without TGF-ß as indicated. After 72 hours, cells were washed and incubated with Propidium Iodide (PI) solution. Binding of PI was measured using fluorescence-activated cell sorting and cell cycle analysis was performed using ModFit LT. The % of cells in G0-G1 phase is depicted as mean % G0-G1 ± standard deviation.

TGF-ß signal transduction is not impaired in cells with oncogenic Ras

First we determined whether HL-60 and TF-1 cells are able to respond to TGF-β or whether the decreased cell cycle arrest is due to the absence of p27. Although oncogenic Ras has been described to be involved in degradation of Smad 4,[178] figure 2A depicts that Smad 4 protein is present in the HL-60 and TF-1 cell lines in comparable levels as in M1 cells.

In addition, TGF-β signal transduction can be inhibited by modulating the cytoplasmic to nuclear translocation of Smad 2/3 through an Erk 1/2-dependent mechanism,[179] which has been described to be activated by Ras in hematopoietic cells.[268] In the N-Ras-containing cells, Smad 3 nuclear

accumulation was observed in response to TGF-β treatment (fig. 2B), indicating that Ras does not interfere with cytoplasmic to nuclear shuttling of Smad 3.
Finally, TGF-β responsive target genes (including Smad 7 and SnoA) were studied by means of Quantitative PCR (Q-PCR) (fig. 2C). In both HL-60 and TF-1 cells, TGF-β treatment resulted in an increased expression of these target genes. This observation was confirmed by a TGF-β reporter assay, where HL-60 and TF-1 cells were transfected with a reporter construct containing 7 copies of a Smad responsive element (7X SBE-luciferase). Upon TGF-β treatment, transactivation of this reporter was observed in both cell lines (fig. 2D). In conclusion these data show that Smad-mediated TGF-β signaling is normal in these N-Ras containing cells.

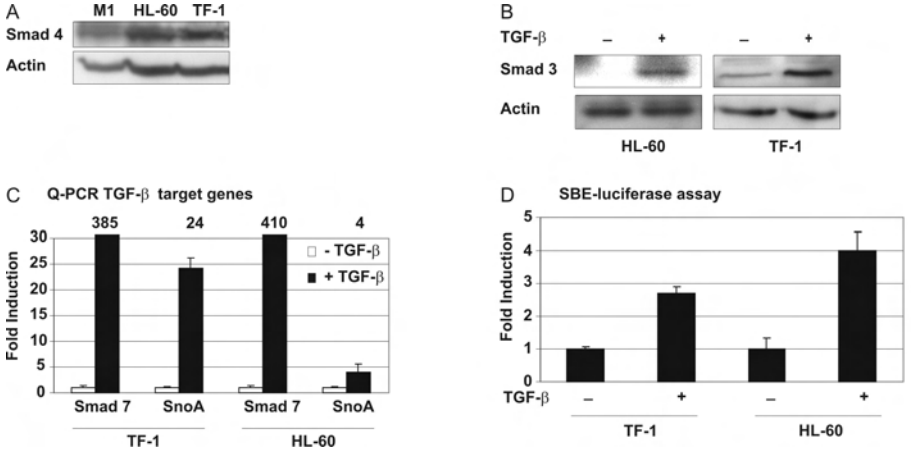

Figure 2 TGF-β signal transduction is normal in N-Ras containing HL-60 and TF-1 cells.
(A) Smad 4 expression in M1, HL-60 and TF-1 cells. After 24 hours of culture, cell extracts were subjected to western blot analysis for Smad 4 and Actin. (B) Smad 3 shows nuclear localization after 30 minutes of TGF-β treatment. HL-60 and TF-1 cells were treated with or without 2 ng/ml TGF-β. Nuclear fractions were isolated and subjected to western blot analysis for Smad 3 and Actin. (C) TGF-β activates transcription of target genes in HL-60 and TF-1 cells. Cells were cultured with 2 ng/ml TGF-β for 1 hour and cDNA was prepared as described. Q-PCR for Smad 7 and SnoA was performed and normalized against expression of the common house hold gene HPRT. Fold inductions are presented as sample means ± standard deviation relative to untreated samples. (D) TGF-β transactivates transcription from a 7X SBE (Smad Binding Element)-luciferase construct. HL-60 and TF-1 cells were electroporated with a 7x SBE Luciferase vector and pDM2-LacZ. After 24 hours, cultures were split in two and treated for 16 hrs with or without 2 ng/ml TGF-β. Cell extracts were subjected to luciferase and β-galactosidase assays as described. Luciferase values were normalized against β-galactosidase activity and fold inductions are presented as sample means ± standard deviation relative to untreated samples.

Oncogenic Ras regulates p27 through a MEK/Erk –dependent pathway

To demonstrate a connection between Ras signal transduction and the absence of p27 expression, TF-1 and HL-60 cells were treated with inhibitors of the Ras (L744832) and MEK/Erk (U0126) pathways. As depicted in figure 3A, a reappearance of p27 expression in TF-1 and HL-60 cells is observed in the presence of inhibitors U0126 and L744832. The effectivity of the inhibitors was confirmed by reduced phosphorylation of Erk 1/2 (fig. 3A and densitometry fig. 3B) and reduced downstream Elk signaling, as investigated by an Elk-Gal4 transactivation assay (fig. 3B).

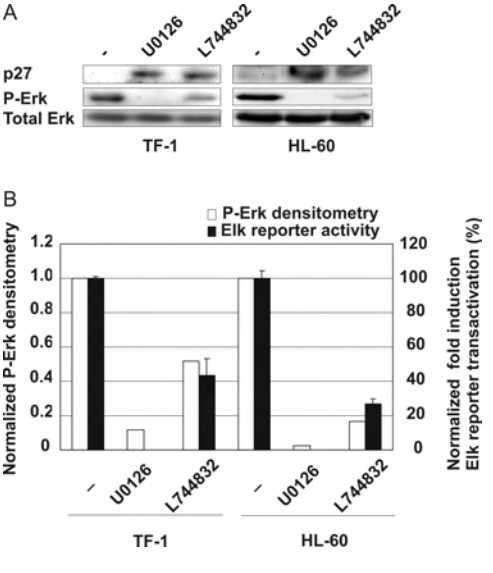

Figure 3 Inhibition of MapK and Ras signal transduction restores p27 levels in TF-1 and HL-60 cells.
(A) Treatment with U0126 and L744832 restores p27 protein levels in TF-1 and HL-60 cells. Cells were cultured for 16 hrs in the presence of 10 μM U0126 or 50 μM L744832. Cell extracts were subjected to western blot analysis for P-Erk, Total Erk and p27. (B) Densitometry of P-Erk and Elk reporter activity indicate inhibition of Ras/ MapK signaling by U0126 and L744832. White bars indicate P-Erk densitometry belonging to fig.3A. Black bars indicate normalized Elk reporter luciferase activity against β-galactosidase activity. HL-60 and TF-1 cells were either mock electroporated or with pGAL4-Elk1, pGAL4-TK-luc and pDM2-LacZ for the Elk-Gal4 transactivation assay. Fold inductions are presented as sample means ± standard deviation relative to untreated samples.

In order to demonstrate that the effect of Ras/MEK/Erk signaling is specific for the p27 protein, additional CDKi's were studied. The CDKi $p21^{Waf1}$ is absent in unstimulated hematopoietic cells,[290] which was confirmed for the HL-60 cell line. Upon treatment with U0126 no change in the expression of p21 protein was observed (fig. 4). TF-1 cells express $p21^{Waf1}$ (fig. 4, first lane), which disappears in the presence of U0126, while no change was observed in the expression of the CDKi $p57^{Kip2}$, as is the same for HL-60 cells (fig. 4). The expression of the CDKi $p16^{INK4a}$ is absent in HL-60 cells, without changes upon U0126 treatment, whereas in TF-1 cells p16 protein is expressed, which appears to decrease slightly upon U0126 treatment (fig. 4). Q-PCR analysis for the expression of the CDKi's $p15^{INK4b}$ and $p18^{INK4c}$ demonstrated no significant changes upon U0126 treatment (data not shown).

Although some changes are observed for p21 and to a lesser extend p16 upon U0126 treatment, the most prominent effect was observed on p21

expression levels which were strongly reduced by U0126 treatment and this would positively affect cell cycle progression. In conclusion, these data strongly suggest that the blockade of TGF-β-induced cell cycle arrest by Ras/MEK/Erk signaling in TF-1 and HL-60 cells is mediated through modulation of the p27 expression levels, since none of the other CDKi's is upregulated after U0126 treatment.

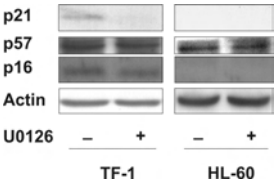

Figure 4 Inhibition of MapK signal transduction does not upregulate other cyclin dependent kinase inhibitors.
The CDKi p21 disappears upon U0126 treatment and the expression of the CDKi's p16 and p57 is not changed upon treatment with U0126. TF-1 and HL-60 cells were cultured for 16 hrs in the presence or absence of 10 μM U0126. Cell extracts were subjected to western blot analysis for p21, p57, p16 and Actin.

TGF-ß-mediated cell-cycle arrest is restored after U0126 and L744832 treatment and is p27 dependent

To demonstrate that the reappearance of p27 is associated with TGF-β mediated cell cycle arrest, TF-1 cells were stimulated with TGF-β in the presence of U0126 or L744832. A significant increase in the percentage of cells in G0-G1 phase (fig. 5A, 13.6 % and 7.2 % respectively, * and ** = $p<0.05$) was demonstrated by FACS analysis.

To study the specificity of this effect, a TF-1 cell culture was generated in which the cells stably express p27 through retroviral transduction with pBabe-Flag-p27. In these cells, cell cycle analyses were performed with TGF-β in the absence and presence of the MEK/Erk inhibitor U0126. Mock (pBabe) transfected TF-1 cells have a minor increase in the percentage of cells in G0-G1 phase after TGF-β treatment (fig. 5B, 7.2 %, $p<0.05$) and this increase can be enhanced by inhibiting the MEK/Erk pathway (20.8 %, ● = $p<0.05$). TF-1 cells expressing pBabe-Flag-p27 already show an almost twofold increase in the percentage of cells in G0-G1 phase (21.6 %, ●● = $p<0.01$) in response to TGF-β, which was not further enhanced by U0126 treatment (18.3 %, ●●● = $p<0.01$) (fig. 5B). These results clearly indicate that the presence of p27 is sufficient to increase the percentage of cells in G0-G1 phase upon TGF-β treatment, although the percentages of untreated cells in G0-G1 phase varied between the different studied groups. Together, these data demonstrate that oncogenic Ras inhibits TGF-β-mediated growth arrest through modulation of p27 levels.

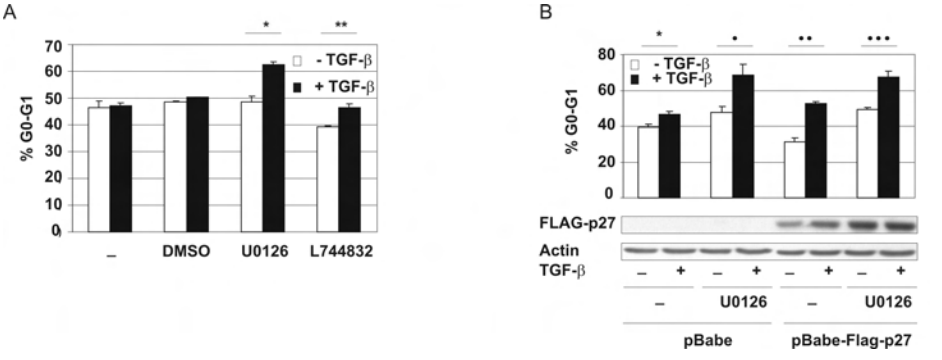

Figure 5 TGF-β-mediated cell cycle arrest is p27 dependent and can be restored by inhibition of MapK and Ras signal transduction.
(A) TGF-β-mediated cell cycle arrest can be restored upon inhibition of the MapK and Ras signal transduction pathways. TF-1 cells were synchronized in M-Phase and subsequently cultured with or without TGF-ß in the presence of DMSO, U0126 or L744832 as described. After 72 hours, cells were washed and incubated with PI solution. Binding of PI was measured using fluorescence-activated cell sorting and cell cycle analysis was performed using ModFit LT. The % of cells in G0-G1 phase is depicted as mean % G0-G1 ± standard deviation. * and ** = p<0.05 (B) Stable overexpression of FLAG-p27 is sufficient to restore TGF-β-mediated cell cycle arrest. TF-1 cells were either mock (pBabe) stable transfected or with pBabe-FLAG-p27 and selected for positive cells with puromycin. The cells were synchronized in M-Phase and subsequently cultured with or without TGF-ß or U0126. After 72 hours, cell cycle analysis was performed as described above. The % of cells in G0-G1 phase is depicted as mean % G0-G1 ± standard deviation. * and ● = p<0.05, ●● and ●●● = p<0.01. Cell extracts were also subjected to western blot analysis for FLAG and Actin.

p27 is not regulated at the transcriptional level, but at the degradation level

p27 protein levels can be regulated at different levels e.g. transcription, translation or degradation.[381] To study how p27 protein levels are modulated, cells were pre-treated with the RNA polymerase II inhibitor Actinomycin D. Although Actinomycin D was effective since it completely blocked TGF-β-induced transcription of Smad 7 (fig. 6B), p27 still reappeared after U0126 and L744832 treatment (fig. 6A, middle panel, compare with left panel, the control for p27 reappearance). No significant upregulation of p27 transcription after U0126 treatment was observed with Q-PCR studies for p27 mRNA, which was confirmed by reporter assays with the p27GL-1609 construct[378] in the TF-1 and HL-60 cells (data not shown).

Treatment with the proteasome inhibitor MG132 induced reappearance of the p27 protein (fig. 6A, right panel), suggesting that oncogenic Ras regulates p27 protein levels through degradation. In view of these data, the mRNA and protein expression levels of SKP2, the E3 ubiquitin ligase of p27, were analyzed. As depicted in figure 6C and D, in the HL-60 and TF-1 cell lines SKP2 expression levels are 4-fold increased as compared to M1 cells.

Subsequently we determined whether Ras signaling enhanced SKP2 levels via an Erk-dependent pathway resulting in p27 degradation. Treatment of HL-60 cells with the Ras inhibitor L744832 resulted in reduced levels of phosphorylated Erk, downregulation of SKP2 expression and the reappearance of p27 protein (fig. 6E). In addition, M1 cells transiently transfected with pCDNA3-Myc-N-Ras^{L61} demonstrated higher SKP2 protein levels than mock transfected control cells (fig. 6F). These data suggest that oncogenic Ras promotes p27 degradation through the activation of Erk and subsequent upregulation of SKP2 expression.

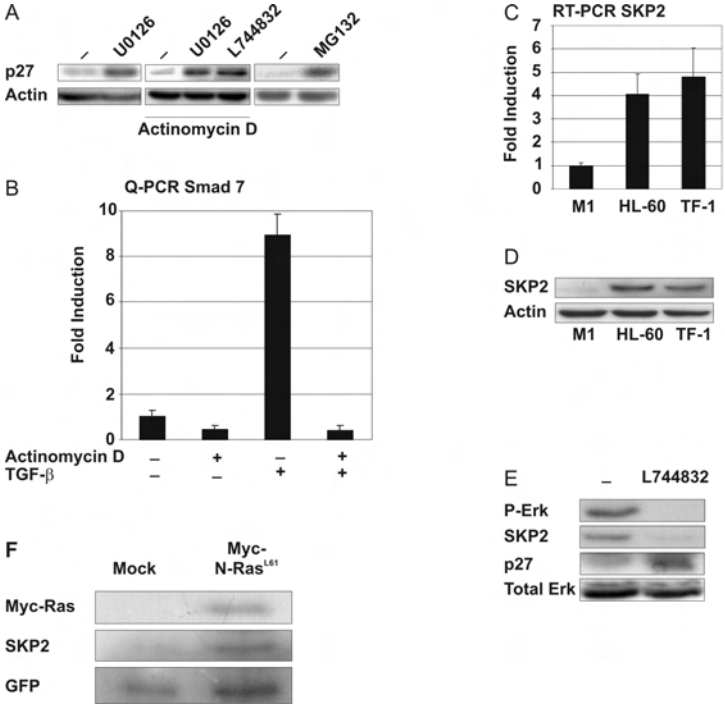

Figure 6 p27 is not regulated at the transcriptional level but at the degradational level in N-Ras containing cells and SKP2 is regulated by a Ras dependent pathway.
(A) Actinomycin D does not affect p27 restoration after U0126 and L744832 treatment and p27 levels are restored after treatment with the proteasome inhibitor MG132. TF-1 cells were cultured for 16 hrs in the presence or absence of U0126, L744832 or 0.25 μM MG132 with or without 0.50 μg/ml Actinomycin D. Cell extracts were subjected to western blot analysis for p27 and Actin. (B) Actinomycin D is effective in blocking TGF-β-induced transcription of Smad 7. TF-1 cells were cultured with 2 ng/ml TGF-β for 1 hour, with or without pre-treatment with 0.50 μg/ml Actinomycin D. cDNA was prepared as described and Q-PCR for Smad 7 was performed and normalized against expression of the common house hold gene HPRT. Fold inductions are presented as sample means ± standard deviation relative to untreated samples. (C) Q-PCR for SKP2 indicates higher mRNA levels in N-Ras containing HL-60 and TF-1 cells. Cells were cultured and cDNA was prepared as described. Q-PCR for SKP2 was performed and normalized against expression of the

common house hold gene HPRT. Fold inductions are presented as sample means ± standard deviation relative to M1 control cells. (D) Western blot analysis for SKP2 indicates higher protein levels in N-Ras containing HL-60 and TF-1 cells. Cells were cultured and cell extracts were subjected to western blot analysis for SKP2 and Actin. (E) Inhibition of Ras signal transduction decreases P-Erk and SKP2 levels and increases p27 levels. HL-60 cells were cultured with or without L744832. After 16 hours, cells were lysed and cell extracts were subjected to western blot analysis for P-Erk, total Erk, SKP2 and p27. (F) Overexpression of Myc-tagged N-Ras^{L61} increases SKP2 levels. M1 cells were transiently co-transfected with GFP and mock or pCDNA3-Myc-N-Ras^{L61}. After 24 hours, GFP+ cells were sorted on a MoFlo cell sorter. Equal amounts of cells were lysed and cell extracts were subjected to western blot analysis for Myc-N-Ras, SKP2 and GFP.

p27 regulation involves degradation

Degradation of p27 has been described to involve two mayor steps, phosphorylation and ubiquitinylation.[363] Phosphorylation of p27 at Threonine 187 has been shown to occur through Cyclin E/CDK2 complexes in the nucleus, followed by binding of the F-box protein and E3 ubiquitin ligase SKP2.[364-368] Phosphorylation of p27 at Serine 10 also has been shown to occur by a yet unknown kinase, followed by nuclear export and degradation in the cytoplasm.[375;376;382-384] In order to investigate the mechanism involved in p27 degradation in N-Ras containing cells, alanine point mutants from known and putative phosphorylation sites, were transiently transfected into TF-1 cells. After 24 hours, cultures were split and treated with U0126 (fig. 7A). Although to slightly different levels, treatment with U0126 resulted in reappearance of wildtype (Wt) and all p27 point mutants proteins. Strikingly, the p27^{T187A} mutant was resistant to degradation in TF-1 cells (fig. 7A, upper and lower panel), indicating that this phosphorylation site is relevant for p27 degradation in the presence of oncogenic Ras.

Subsequently, the degradation of p27 was studied in more detail. Virally transduced Flag-p27 TF-1 cells were treated with both the proteasome inhibitor MG132 and the translation inhibitor cycloheximide. This provides the opportunity to investigate steady state levels of p27 in the cell. In addition, it is possible to study whether p27 is phosphorylated and whether degradation of p27 is a nuclear event.

After 18 hours of culture, p27, phosphorylated at serine 10 and threonine 187, was confined to the nuclear compartment without modulation by U0126 treatment (fig. 7B). In addition, Flag-p27 was only detected in the nuclear compartment (fig. 7B), suggesting that the degradation of p27 occurs solely in the nucleus and no nuclear to cytoplasmic translocation takes place. The box next to panel B demonstrates the absence of p27 in both nuclear and cytoplasmic fractions in untreated parental TF-1 cells. M1 cells are shown as control. Moreover, these findings implicate that the phosphorylation of T187 is a pre-requisite for p27 degradation, but it is unlikely to be the cause of degradation. It is more conceivable that the elevated SKP2 levels are

connected with the increased degradation of p27. Subsequently, co-immunoprecipitation studies were performed in the virally transduced Flag-p27 TF-1 cell line, to demonstrate complex formation between p27 and SKP2. Indeed, p27 can be precipitated with SKP2 (fig. 7C, left panel), and vice versa SKP2 precipitates with p27 (right panel), indicating that these two proteins are in the same complex in the TF-1 cell line.

Finally, to study whether SKP2 is involved in p27 degradation in N-Ras containing cells, RNA interference (RNAi) studies for SKP2 were performed in the TF-1 cell line. Knockdown of SKP2 resulted in a reappearance of p27 protein, without an effect on GAPDH or actin levels (fig. 7D). Conversely, short interfering RNA (siRNA) oligos for GAPDH only affect GAPDH expression levels and had no effect on p27. These data further support a role for SKP2 in p27 degradation.

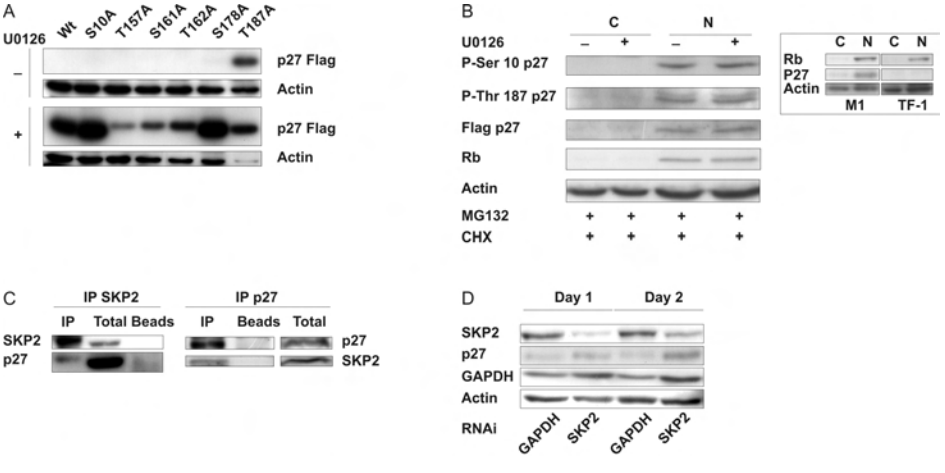

Figure 7 p27 protein is degraded in a Phospho Threonine 187/SKP2-dependent manner.
(A) Mutant $p27^{T187A}$ is resistant to degradation. TF-1 cells were electroporated with pCMV Tag2B or pCDNA3 FLAG wt p27 or S10A, T157A, S161A, T162A, S178A or T187A mutant p27. After 24 hours, cultures were split in two and for 16 hrs treated with or without U0126. Cell extracts were subjected to western blot analysis for FLAG and Actin. (B) p27 is phosphorylated on S10 and T187 and is specific nuclear localized. Stable expressing pBabe-FLAG-p27 TF-1 cells were cultured for 16 hrs in the presence of 0.25 μM MG132 and 100 μg/ml Cycloheximide (CHX) with or without 10 μM U0126. Cytoplasmic and nuclear fractions were isolated and subjected to western blot analysis for Flag p27, phospho Ser10 p27, phospho Thr187 p27, Rb and Actin. The box demonstrates the absence of p27 from both nuclear and cytoplasmic fractions in TF-1 cells. M1 cells are shown as a control. (C) SKP2 and p27 form a complex in N-Ras containing TF-1 cells. Stable expressing pBabe-FLAG-p27 TF-1 cells were cultured for 16 hrs and cell extracts were subjected to co-immunoprecipitation (IP) studies between SKP2 and p27 as described in material and methods. The precipitates were subjected to western blot analysis for SKP2 or p27. IP indicates samples subjected to IP procedure, total indicates samples before IP procedure and beads indicates IP procedure without antibody to exclude aspecific binding (D) p27 protein is reappearing after RNA interference for SKP2. TF-1 cells were transfected with siRNA oligos for SKP2 and GAPDH as described and at the indicated time points cell were lysed and extracts were subjected to western blot analysis for SKP2, p27, GAPDH and Actin.

Discussion

Loss of responsiveness to anti-proliferative signals of growth factors, such as TGF-ß, might provide a growth advantage to malignant cells.[385] Here we show that leukemic cells that carry an oncogenic N-Ras^{L61} mutation escape TGF-ß-mediated cell cycle arrest through nuclear degradation of the cyclin dependent kinase inhibitor p27$^{Kip1/Waf1}$ in a Ras/MEK/Erk/SKP2-dependent manner. This finding highlights an alternative pathway that results in TGF-β unresponsiveness. Among these alternative pathways are phosphorylation of Smad 3 and subsequent inhibition of nuclear translocation of this protein,[179] the degradation of Smad 4[178] and mislocalization of p27 to the cytoplasm.[171]

In this study we demonstrate that cells with N-Ras mutations have elevated SKP2 protein levels, a link that has been observed before.[386] In other cellular settings SKP2-independent regulation of p27 has been reported, that coincided with Erk activation and involved translocation of p27 to the cytoplasm.[387] Phosphorylation on different residues has been demonstrated to be involved in cytoplasmic localization and or stabilization of p27, e.g. Serine 10, Threonine 157 and Serine 178.[372;374;375;388] In our experiments we observed that alanine point mutants of S10 and S178 are upregulated to higher levels then other mutants by U0126 treatment (fig. 7A), suggesting additional cytoplasmic degradation routes of p27. However, we show that p27 is not translocated to the cytoplasm, even though p27 is phosphorylated on residue S10 (fig. 7B). The observed differences in expression between the various alanine point mutants are therefore likely to be the results of different transfection efficiencies. The only phosphorylation mutant that was resistant to degradation was the p27^{T187A} mutant, suggesting SKP2-dependent nuclear degradation.[363-368;375;376;384] However, inhibition of the MEK/Erk pathway did not modulate the phosphorylation of the T187 residue, suggesting that p27 phosphorylation is a pre-requisite for binding to SKP2, but not the primary cause for the degradation process. Upregulation of SKP2 expression and binding to p27 in the nucleus seems to be the primary cause of the increased degradation of p27. The observed difference between our results and additional studies[387] is therefore likely to be cell type dependent.

Recently, TGF-β was demonstrated to destabilize SKP2 by ubiquitin-mediated proteolysis and decreased levels of Cks1 mRNA.[389] This in turn stabilized the p27 protein and resulted in cell cycle arrest. In our study, however, TGF-β treatment of wt TF-1 cells did not lead to an increase in p27 levels, probably because oncogenic Ras upregulates SKP2 to such levels, that the reduction of SKP2 by TGF-β is not sufficient to prevent p27 degradation by

SKP2. Although it is reported that deletion of p27 causes hyperproliferation,[390] overexpression of FLAG-p27 itself is not enough to induce growth arrest in TF-1 cells. This suggests the activation of an additional signal transduction pathway by TGF-β. Alternatively, there is a persistent disbalance in the SKP2 and p27 levels, resulting in an inappropriate activation of cell cycle progression. In the stable FLAG-p27 cells an increased expression of p21 was observed and in some experiments p15 (data not shown). Since p27 has been demonstrated to enhance Sp1 stability and promoter binding[391] and both p21 and p15 expression is enhanced by Sp1,[392] the upregulation of p21 and p15 could be an effect of p27-mediated Sp1 activation. These two CDKi's might contribute to p27-mediated growth arrest in response to TGF-β, but they are downstream of p27.

P27 protein degradation is complex and controlled by various mechanisms. In this report we show that p27 protein levels are regulated by degradation through a T187 phosphorylation-dependent, MEK/Erk-dependent, SKP2 pathway. Different signaling pathways downstream of MEK/Erk have been identified that affect SKP2 expression. Cul1, a critical component of the SCF(SKP2) ubiquitin ligase is a direct transcriptional target of c-Myc.[393] It has been demonstrated that Myc expression and activity is downregulated upon MEK1 inhibition in conjunction with upregulation of p27.[394] Indeed, inhibition of Ras activity with L744832 resulted in decreased c-Myc protein levels (data not shown), in accordance with reduced SKP2 levels and restoration of p27 levels (fig. 6E). Alternatively, the SKP2 gene has been demonstrated to be a transcriptional target of GA binding protein (GABP) [395] and DNA micro array studies have indicated that GABP is a target gene of MEK/ERK signaling.[396] Both possibilities support our findings that (1) MEK/Erk signaling does not directly influence the phosphorylation status of p27 nor the transcription of p27; and that (2), the delayed upregulation of p27 upon inhibition with U0126 and L744832 indicates an indirect effect of MEK/Erk signaling.

The inhibitory effect of p27 on the cell cycle is not restricted to the effects of TGF-β. Similar findings have been observed in solid tumors in response to vitamin D and retinoic acid.[397;398] Since N-Ras transformed teratocarcinoma and prostate epithelial cells are resistant to retinoic acid,[399;400] the above described mechanism of disturbed p27 degradation widens the extend of enhancing the proliferative capacity of cells bearing oncogenic Ras mutations. Recently, studies demonstrated that unfavorable treatment results in AML were associated with elevated SKP2 levels and with a high cytoplasmic to nuclear p27 ratio.[155;401] Although in our model cytoplasmic p27 was not observed, the high cytoplasmic to nuclear ratio in these studies might be due to nuclear

degradation of p27 in a SKP2-dependent manner thereby increasing the cytoplasmic p27 relative to the nuclear p27 levels. Ras-GTP and downstream targets can modulate these pathways in several ways, since Ras activation can be triggered directly by mutations in the Ras oncogene, or indirectly by mutations in the PTPN11 gene, encoding for the protein tyrosine phosphatase SHP-2[94] (and references therein), indicating the complexity of the mechanisms leading to oncogenic transformation and proliferation.

Recently, different phase I-II studies have been initiated to interfere with Ras-mediated signaling in AML patients and to increase the susceptibility of the leukemic cells for the cytotoxic effects of chemotherapy.[402-404] This report indicates that this is not the only advantage of this approach. AML cells might also become more susceptible for negative regulatory molecules that limit cell proliferation, including TGF-β.

In conclusion, we demonstrate that hematopoietic cells with N-Ras mutations do not respond to the inhibitory effects of TGF-β on cell cycle progression, as a result of the absence of p27. This is due to a constitutive nuclear degradation of p27 in a Ras/MEK/Erk/SKP2-dependent manner.

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STAT5 is required for long-term maintenance of normal and leukemic human stem/progenitor cells

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Chapter 7

Abstract

The transcription factor STAT5 fulfills a distinct role in the hematopoietic system, but its precise role in primitive human hematopoietic cells remains to be elucidated. Therefore, we performed STAT5 RNAi in sorted Cord Blood (CB) and Acute Myeloid Leukemia (AML) $CD34^+$ cells by lentiviral transduction and investigated effects of STAT5 downmodulation on the normal stem/progenitor cell compartment and the leukemic counterpart. STAT5 RNAi cells displayed growth impairment, without affecting their differentiation in CB and AML cultures on MS5 stroma. In CB, limiting dilution assays demonstrated a 3.9-fold reduction in progenitor numbers. Stem cells were enumerated in Long-Term Culture-Initiating Cell assays and the average LTC-IC frequency was 3.25-fold reduced from 0.13% to 0.04% by STAT5 downregulation. Single-cell sorting experiments of CB $CD34^+/CD38^-$ cells, demonstrated a 2-fold reduced cytokine-driven expansion, with a subsequent 2.3-fold reduction of progenitors. In sorted $CD34^+$ AML cells with constitutive STAT5 phosphorylation (5/8) STAT5 RNAi demonstrated a reduction in cell number (72%±17%) and a decreased expansion (17±15 vs. 80±58 in control cultures) at week 6 on MS5 stroma. Together, our data indicate that STAT5 expression is required for the maintenance and expansion of primitive hematopoietic stem and progenitor cells, both in normal as well as leukemic hematopoiesis.

Introduction

The hematopoietic environment is a highly dynamic system, where multipotent hematopoietic stem cells (HSCs) perpetually make decisions regarding self-renewal, lineage commitment and terminal differentiation in order to provide sufficient numbers of mature blood cells. An important transcription factor within the hematopoietic system, involved in self-renewal, proliferation and apoptosis in response to a wide range of cytokines, is Signal Transducer and Activator of Transcription 5 (STAT5).[217;225;226] Two isoforms of this STAT family member have been cloned, STAT5A and B, and like other STAT proteins STAT5 is a latent cytoplasmic transcription factor that dimerizes upon phosphorylation by a (cytokine) activated receptor complex. The activated STAT complex then translocates to the nucleus where it binds to specific DNA sequences and increases transcription of target genes.[227]

In homozygous STAT5AB knock-out mice, the presence of an almost normal peripheral blood count suggested that STAT5 is either not necessary or redundant for adult hematopoiesis, even though colony assays demonstrated reduced numbers of cytokine-responsive myeloid progenitors.[239] Instead, STAT5 was assigned a role in fetal liver and stress erythropoiesis due to Bcl-X_L –mediated anti-apoptosis.[405;406] In competitive repopulation experiments however, STAT5AB-null cells demonstrated a severe impairment in engrafting wild-type recipients, which did not appear to be due to a homing deficiency, but rather to a failure in post-engraftment hematopoiesis.[240;241] Also, the cytokine responsiveness was severely impaired in STAT5AB knockout cells.[238] Gain-of-function studies demonstrated that constitutively activated STAT5[1*6] imposed a self-renewal phenotype on human Cord Blood (CB)-derived $CD34^+$ stem and progenitor cells.[37] These observations were consistent with overexpression data in murine HSCs, where the self-renewal phenotype was coupled to the development of a myeloproliferative disease (MPD).[36]

In acute myeloid leukemia (AML) constitutive activation of STAT5 has been demonstrated in approximately 66% of the cases[40;234] and may be attributed to activating mutations in upstream kinases, such as FLT3[235], KIT[236]or JAK2[237] or alternatively be due to autocrine growth factor production.[40] This prompted us to ask whether AML cells are dependent upon STAT5 activation for their survival and proliferation. Recently, we have described an improved method for long-term culture of $CD34^+$ AML cells. In this assay expansion, leukemic cobblestone area (L-CA) formation and self-renewal can be studied *in vitro* up to 22 weeks (van Gosliga *et al.,* submitted manuscript). In the present study, this assay was combined with an optimized lentiviral transduction protocol in

order to introduce STAT5 RNAi hairpins into these expanding AML cells and the results were compared with normal $CD34^+$ CB cells.

Our data indicate that lentiviral-mediated RNA interference of STAT5 in CB $CD34^+$ cells reduced the growth rate of primitive stem- or progenitor cells, leading to decreased CFC and LTC-IC numbers and eventually resulted in lower expansion rates. Single cell assays with the more primitive CB $CD34^+/CD38^-$ cell population suggested that STAT5 is necessary for maintenance of hematopoiesis. Subsequent introduction of the STAT5 RNAi hairpin into $CD34^+$ AML cells, a fraction enriched for L-CA forming (stem) cells, demonstrated that self-renewal and expansion of leukemic stem cells was also dependent upon STAT5 expression. Thus, we conclude that STAT5 expression is required for the maintenance and expansion of human stem/progenitor cells in both normal and leukemic hematopoiesis.

Materials and methods

Long-term cultures on stroma - Cord blood (CB) $CD34^+$ cells were derived from neonatal cord blood from healthy full-term pregnancies after informed consent from the Obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands and isolated by MiniMACS (Myltenyi Biotec) selection. After transduction, 30.000 cells were expanded on MS5 stromal cells in Long Term Culture (LTC) medium (αMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% Horse serum (Sigma, Zwijndrecht, The Netherlands), penicillin and streptomycin, 200 mM Glutamine, 57.2 µM β-mercaptoethanol (Sigma) and 1 µM hydrocortisone (Sigma). AML blasts from peripheral blood cells or bone marrow cells from untreated patients with AML were studied after informed consent and the protocol was approved by the Medical Ethical Committee. AML mononuclear cells were isolated by density gradient centrifugation and $CD34^+$ cells were selected by MiniMACS. After transduction, 200.000 cells were plated onto T25 flasks pre-plated with MS5 stromal cells. AML cells were expanded in LTC medium supplemented with 20 ng/ml Interleukin 3 (IL-3; Gist-Brocades, Delft, The Netherlands), Granulocyte Colony Stimulating Factor (G-CSF; Rhone-Poulenc Rorer, Amstelveen, The Netherlands) and Thrombopoietin (TPO; Kirin, Japan). Cultures were kept at 37ºC and 5% CO_2. Cultures were demidepopulated weekly for analysis. TF-1 cells were cultures in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% FCS and 10 ng/ml GM-CSF (Genetics Institute, Cambridge, MA, USA).

Flow cytometry analysis - All fluorescence activated cell sorter (FACS) analyses were performed on a FACScalibur (Becton Dickinson (BD), Alpen a/d Rijn, The Netherlands) and data was analyzed using WinList 3D (Topsham, USA). Cells were sorted on a MoFlo (DakoCytomation, Carpinteria, CA, USA). Antibodies were obtained from BD.

Lentiviral transductions – $2.5x10^6$ 293T Human Embryonic Kidney cells were transduced with 3 µg pCMV Δ8.91, 0.7 µg VSV-G and 3 µg of pTRIP Renilla RNAi or pTRIP STAT5 RNAi (kind gifts of Prof. Dr. H. Spits, Department of Cell Biology and Histology, Amsterdam Medical Center, Division of Immunology, Netherlands Cancer Institute, Amsterdam. Target sequences have been described by Scheeren et al.[407]). After 24 hours medium was changed to HPGM (Cambrex, Verviers, Belgium) and after 12 hours supernatant containing lentiviral particles was harvested and stored at -80ºC. $CD34^+$ AML blasts and CB $CD34^+$ cells were isolated with MiniMACS columns and subsequently cultured in RPMI supplemented with 10% FCS, 20 ng/ml IL-3, G-CSF and TPO for 4 hours at 37ºC and 5% CO_2 or in HPGM supplemented with c-Kit ligand), Flt-3 ligand (both from Amgen, USA) and TPO (100 ng/ml each) for 16 hours at 37ºC and 5% CO_2 respectively. AML blasts were transduced in 3 consecutive rounds of 8 to 12 hours with lentiviral supernatant supplemented with 10% FCS, 20 ng/ml IL-3, G-CSF and TPO, and Polybrene (4 µg/ml; Sigma) and CB $CD34^+$ cells in 2 consecutive rounds of 8 to 12 hours with lentiviral supernatant supplemented with c-Kit Ligand/Flt-3 ligand/TPO (100 ng/ml each) and Polybrene (4 µg/ml). TF-1 cells were transduced in 1 round of 12 hours with lentiviral supernatant supplemented with 10% FCS, 10 ng/ml GM-CSF and Polybrene (8 µg/ml). Transduction efficiency was measured by FACS analysis and knock-down was investigated by means of Western blot using antibodies against phospho STAT5A/B Tyr 694/699 (Millipore, Bedford, MA, USA), STAT5 (C17) and STAT3 (C20) (Santa Cruz, CA, USA) and Actin (C4) (ICN Biomedicals, Zoetermeer, The Netherlands) in a 1:1000 dilution in PBS on an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA) or using ECL according to the manufacturer's instructions (Roche Diagnostics, Almere, The Netherlands). Alternatively, knock-down was investigated by means of quantative RT-PCR (Q-PCR, sequences and conditions are available on request).

CFC, CFU-GM, BFU-E and LTC-IC assays - CFC assays and LTC-IC assays on MS5 stromal cells were performed as described previously.[408] Briefly, CFC

assays were performed in 1.2% methylcellulose containing 30% FCS, 57.2 µM β-mercaptoethanol, and 2 mM Glutamine, supplemented with 20 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml G-CSF, 20 ng/ml c-Kit ligand and 6 U/ml EPO (Cilag: Eprex; Brussels, Belgium). Cells were either plated in bulk by 1000 transduced cells per plate in duplicate or in 96-well plates in 1, 3, 9, 27, 81, or 243 cells per well. CFU-GM and BFU-E assays were performed in 1.2% methylcellulose containing 30% FCS, 57.2 µM β-mercaptoethanol, and 2 mM Glutamine, supplemented with 10 ng/ml GM-CSF and 10ng/ml IL-3 or 2 U/ml EPO respectively. LTC-IC assays were performed by plating transduced CB $CD34^+$ cells in limiting dilutions in the range of 10-2400 cells per well on MS5 stromal cells in 96-well plates in LTC medium.

Single cell assays – CB $CD34^+$ cells were isolated using MiniMacs columns and cultured in HPGM supplemented with c-Kit Ligand/Flt-3 ligand/TPO (100 ng/ml each) for 8 hours at 37ºC and 5% CO_2 and subsequently transduced in 1 round of 8 hours with lentiviral supernatant supplemented with c-Kit Ligand/Flt-3 ligand/TPO (100 ng/ml each) and Polybrene (4 µg/ml). $CD34^+$ $CD38^-$ YFP/GFP^+ cells were single sorted into 96-well plates into 100 µl HPGM supplemented with c-Kit Ligand/Flt-3 ligand/TPO (100 ng/ml each) and followed microscopically for 100 hrs. After this period, methylcellulose for CFC assays was added and colonies were scored after two weeks.

Results

STAT5 downregulation impairs long-term expansion of CB $CD34^+$ cells on MS-5 bone marrow stroma

In order to address whether downmodulation of STAT5 expression affects the proliferation and survival of human stem- and progenitor cells, CB $CD34^+$ cells were sorted and transduced with lentiviral vectors containing short interfering hairpins against control (Renilla luciferase) or STAT5 mRNA (fig.1A). Flow cytometric analysis demonstrated transduction efficiencies ranging between 50% and 95% (fig. 1B and data not shown). Western blot analysis confirmed efficient downregulation of STAT5 in CB $CD34^+$, up to 70%-75% down modulation relative to STAT5 expression in control cells (fig. 1C, normalized against STAT3 protein, with control cultures arbitrarily set at 100%). STAT3 protein, as a close homologue of STAT5, is shown to indicate specificity of the STAT5 hairpin. Knock-down of STAT5 protein levels correlated well with downregulation of STAT5 mRNA levels in transduced cells (fig. 1D, STAT5

mRNA values were normalized against GAPDH mRNA values). In addition, in TF-1 cells transduced with STAT5 hairpins it was demonstrated that phospho-STAT5 was comparably downregulated as total STAT5 (fig.1C).

To evaluate the effects of STAT5 knockdown on $CD34^+$ cell growth and differentiation, transduced cells were plated onto MS-5 stromal cells and cultures were weekly demidepopulated and analyzed for 5 weeks by flow cytometry. No effects of STAT5 downmodulation were observed on the myeloid differentiation program, as no change in the percentage of GFP^+ cells positive for CD14 (40%±6% vs. 46%±5%), CD15 (47%±11% vs. 38%±14%) and CD11b (65%±3% vs. 75%±3%) was observed (fig. 1E, n=3). STAT5 downmodulation also did not affect erythroid differentiation, since the percentage of cells positive for CD36, CD71 and Glycophorin A was not different from control Renilla transduced cells (data not shown).

The percentage of YFP/GFP^+ cells in suspension was also monitored for 5 weeks. Stable YFP percentages were observed over time in control Renilla cultures relative to the YFP percentage at week 1, indicating no negative effects of the transduction procedures (fig. 1F). The STAT5 hairpin (GFP), however, induced a pronounced reduction of GFP^+ cells in suspension over time (29% ± 12.7%), indicating that STAT knockdown impairs cell survival or proliferation. To test whether increased apoptosis was responsible for the reduction in cell numbers, AnnexinV-APC labeling was performed. No difference in apoptosis within the YFP/GFP^+ population between control and STAT5 RNAi MS-5 co-cultures was demonstrated (fig. 1G), as well as in $CD34^+$ cells cultured in 100 ng/ml c-Kit Ligand/Flt-3 ligand/TPO (data not shown).

Next, we tested whether changes in cell-cycle status could account for the differences observed between Renilla YFP^+ and STAT5 GFP^+ cells. Figure 1H shows Hoechst labeling of YFP^+ and GFP^+ cells (from MS5 co-cultures), demonstrating approximately 61% of cells in G1 phase, 30% of cells in S-phase and 9% of cells in G2/M phase in both cultures Similar results were obtained with c-Kit Ligand/Flt-3 ligand/TPO treated cultures (data not shown). Together, the reduced growth of STAT5 knockdown cultures and the absence of differences in both apoptosis and cell-cycle assays, suggest that knockdown of STAT5 impairs the outgrowth of a small fraction of primitive stem-, or progenitor cells.

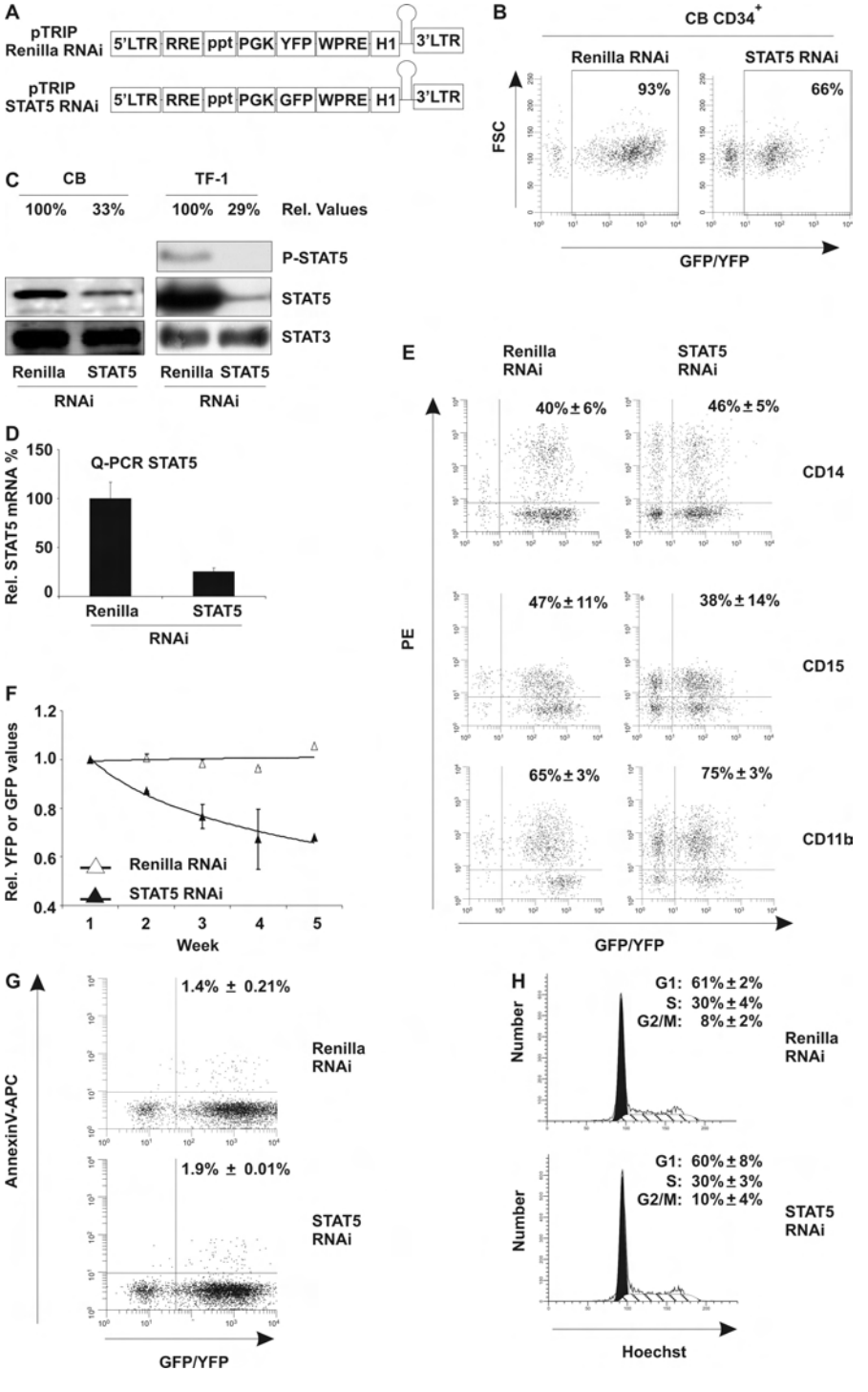
A
pTRIP Renilla RNAi
5'LTR RRE ppt PGK YFP WPRE H1 3'LTR
pTRIP STAT5 RNAi
5'LTR RRE ppt PGK GFP WPRE H1 3'LTR
B
CB CD34+
Renilla RNAi
STAT5 RNAi
93%
66%
FSC
GFP/YFP
C
CB
TF-1
100% 33%
100% 29%
Rel. Values
P-STAT5
STAT5
STAT3
Renilla STAT5
Renilla STAT5
RNAi
RNAi
D
Q-PCR STAT5
Rel. STAT5 mRNA %
150
100
50
0
Renilla
STAT5
RNAi
E
Renilla RNAi
STAT5 RNAi
40%±6%
46%±5%
CD14
47%±11%
38%±14%
CD15
65%±3%
75%±3%
CD11b
PE
GFP/YFP
F
Rel. YFP or GFP values
1.2
1.0
0.8
0.6
0.4
Renilla RNAi
STAT5 RNAi
1 2 3 4 5
Week
G
AnnexinV-APC
1.4% ± 0.21%
Renilla RNAi
1.9% ± 0.01%
STAT5 RNAi
GFP/YFP
H
Number
G1: 61%±2%
S: 30%±4%
G2/M: 8%±2%
Renilla RNAi
G1: 60%±8%
S: 30%±3%
G2/M: 10%±4%
STAT5 RNAi
Hoechst

Figure 1 Downmodulation of STAT5 by RNAi decreases growth of CD34$^+$ stem/progenitor cells.
(A) Schematic overview of the lentiviral RNAi vectors used in these studies. (B) Transduction efficiency of CD34$^+$ CB stem/progenitor cells, performed as indicated in Material and methods (M&M) (C) Western blot analysis of STAT5 levels in control (Renilla) or STAT5 RNAi transduced CD34$^+$ cells. Percentages indicate expression of STAT5 relative to control cells, normalized against STAT3 levels. Phospho STAT5 levels are shown for the hematopoietic TF-1 cell line. Quantitative western blot analysis was performed on an Odyssey infrared scanner or using Quantity One imaging software from BioRad (Veenendaal, The Netherlands). (D) Quantative PCR for STAT5 mRNA in transduced CB CD34$^+$ stem/progenitor cells. STAT5 mRNA was normalized against GAPDH mRNA expression. (E) FACS analysis of transduced CB cells on MS5 co-culture. MS5 co-cultures were analyzed at week 3 for cells positive for CD14, CD15, and CD11b. The average percentage (from 3 independent experiments) of YFP/GFP$^+$ cells positive for the respective markers is shown with standard deviations. (F) Transduced CD34$^+$ CB cells in long-term co-culture on MS5 stromal cells. Shown are average YFP (Renilla control) or GFP STAT5 RNAi percentages relative to week 1. A trendline is indicated and error bars denote standard deviations. N = 4. (G) AnnexinV-APC stain of transduced CB cells on MS5 co-culture. The average percentage of AnnexinV positive cells within the YFP or GFP gate from 3 independent experiments is shown with standard deviations. (H) Hoechst (5µg/ml) cell-cycle labelling of YFP$^+$ or GFP$^+$ CB cells on MS5 co-culture. The average percentages of cells in G1, S or G2/M phase of the cell cycle are shown from 3 independent experiments with standard deviations.

STAT5 down-regulation reduces progenitor and stem cell frequencies

To test the effects of STAT5 downmodulation on the progenitor pool, colony assays were performed with transduced CB CD34$^+$ cells in methylcellulose. Down-modulation of STAT5 levels resulted in a 56.5% ± 25% reduction of Colony Forming Unit- Granulocyte Macrophage (CFU-GM) colonies (P= 0.01; n=3) and a 43.3% ±31.9% reduction of Burst Forming Unit-erythrocyte (BFU-E) colonies (P< 0.05; n=3). In fig. 2A a representative example is shown. The reduction after STAT5 RNAi of total colony numbers in Colony Forming Cell (CFC) assays relative to Renilla RNAi was 43.3% ±16.2% (P< 0.01; n=3, data not shown).

The precise progenitor frequency was determined in limiting dilution CFC-assays using YFP en GFP-sorted cells. Knockdown of STAT5 in CD34$^+$ cells demonstrated an almost 4-fold reduction in the frequency of progenitors from 5.42% ± 0.47% in Renilla control cells to 1.37% ± 0.13% in STAT5 knockdown (fig. 2B and 2C. P< 0.02).

In order to investigate whether Long-Term Culture- Initiating Cell (LTC-IC) frequencies might also be affected, limiting dilution LTC-IC assays were performed with YFP and GFP sorted cells in 3 independent experiments. Data in figure 2D and E demonstrate a reduction in the percentage of LTC-IC colonies formed from 0.23% to 0.037% upon knockdown of STAT5. In two additional independent experiments a reduction from 0.044% to 0.023% and from 0.1% to 0.048% was observed (Renilla versus STAT5 RNAi, respectively). On average this indicates a 3.25-fold reduction in LTC-IC frequency from 0.13% to 0.04% upon STAT5 RNAi. This decrease in the number of LTC-IC colonies after STAT5 RNAi was also observed when enumerating the number

of Cobblestone Area Forming Cells (CAFCs; Renilla versus STAT5 respectively 0.097% and 0.020%, data not shown). Together these data suggest that a reduction of STAT5 leads to an impaired maintenance of stem and progenitor cells.

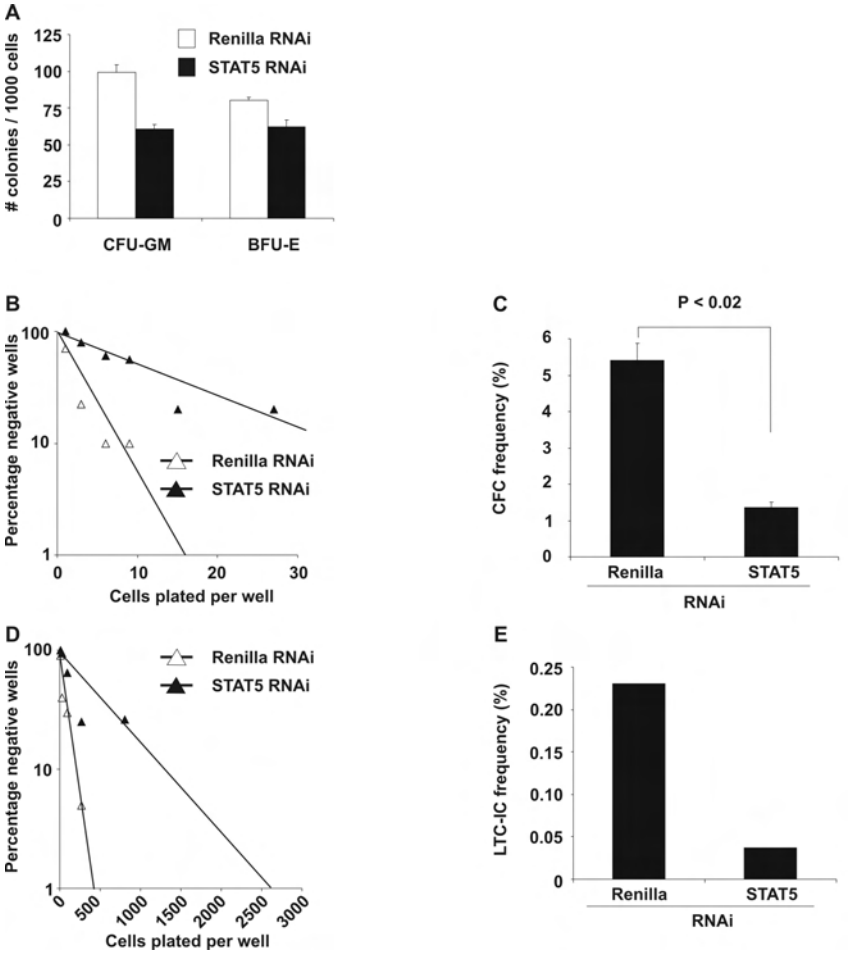

Figure 2 Knockdown of STAT5 reduces CFC and LTC-IC frequencies of CB CD34$^+$ cells.
(A) CFU-GM and BFU-E assays with control and STAT5 transduced CB cells. Assays were performed 3 times in duplicate under conditions indicated in M&M. Error bars denote standard deviations. (B) Limiting dilution CFC assay performed as described in M&M. Poisson statistics were used to calculate CFC progenitor frequencies. N=3, a representative experiment is shown. (C) Percentage of CFC-progenitors as calculated from the limiting dilution assays. The average of 3 experiments is shown, with error bars representing standard deviations. A student t-test was performed to calculate statistical differences between the two groups. (D) Limiting dilution LTC-IC assay performed as described in M&M. Poisson statistics were used to calculate LTC-IC frequencies. N=3, a representative experiment is shown. (E) Percentage of LTC-IC cells as calculated from the limiting dilution experiment shown in C. N=3.

Knock-down of STAT5 decreases the production of offspring of CD34$^+$/CD38$^-$cells

Apoptosis and the cell cycle distribution analysis did not show a change in the majority of the CD34$^+$ cells (fig. 1G and H). However, since both stem cell (LTC-IC) and progenitor frequencies decreased in STAT5 knockdown cells (fig. 2A-DE), we wondered what the effect of STAT5 RNAi would be in a subpopulation of CD34$^+$ cells, e.g. primitive CD34$^+$/CD38$^-$ cells, a fraction which is highly enriched for cells with stem cell activity. [409] After transduction of CD34$^+$ isolated cells, CD34$^+$/CD38$^-$ YFP$^+$ (Renilla) and GFP$^+$ (STAT5) cells were sorted single-cell into 96-wells plates (Fig. 3A) and subsequently monitored for 100 hours by microscopic evaluation. The number of cells present per well were enumerated each 25 hours and classified “quiescent” when still containing only 1 cell and “proliferating” when multiple cells were observed. Figure 3B demonstrates that the number of quiescent cells was marginally increased in STAT5 knockdown cells while the number of wells that contained proliferating cells was slightly decreased, but both changes were not significant ($P>0.1$, n=3). However, of the CD34$^+$/CD38$^-$ cells that were capable of division, wells containing STAT5 knockdown cells demonstrated significantly less cells after 100 hours of culture than control wells (fig. 3C, 8 ± 8 vs. 16 ± 7 cells respectively, $P< 1*10^{-6}$, 30 individual cells for each group are shown). Furthermore, the average number of daughter cells produced in each well by STAT5 knockdown cells was significantly decreased compared to control Renilla knockdown cells (fig. 3D, respectively 5.6 ± 0.9 cells vs.7.1 ± 0.9 cells, $P< 0.01$), resulting in a lower total amount of cells per plate (289 ± 64 vs. 198 ± 72 cells, respectively for Renilla and STAT5 RNAi, $P< 0.01$, data not shown). In addition, methyl cellulose was added to these wells after 100 hours of culture and after two weeks the number of CFC colonies was enumerated. The number of wells positive for colonies was 2.3-fold lower in STAT5 hairpin transduced cells compared to control cells (36 ± 6.5 vs. 16 ± 2.9 colonies, respectively for Renilla and STAT5 RNAi, $P< 0.05$, n=3, fig. 3E), which verified the outcome of the limiting dilution CFC assays (fig. 2B and C). Together these data demonstrate that at the single cell level in primitive CD34$^+$/CD38$^-$ cells, STAT5 RNAi impairs the proliferation rate.

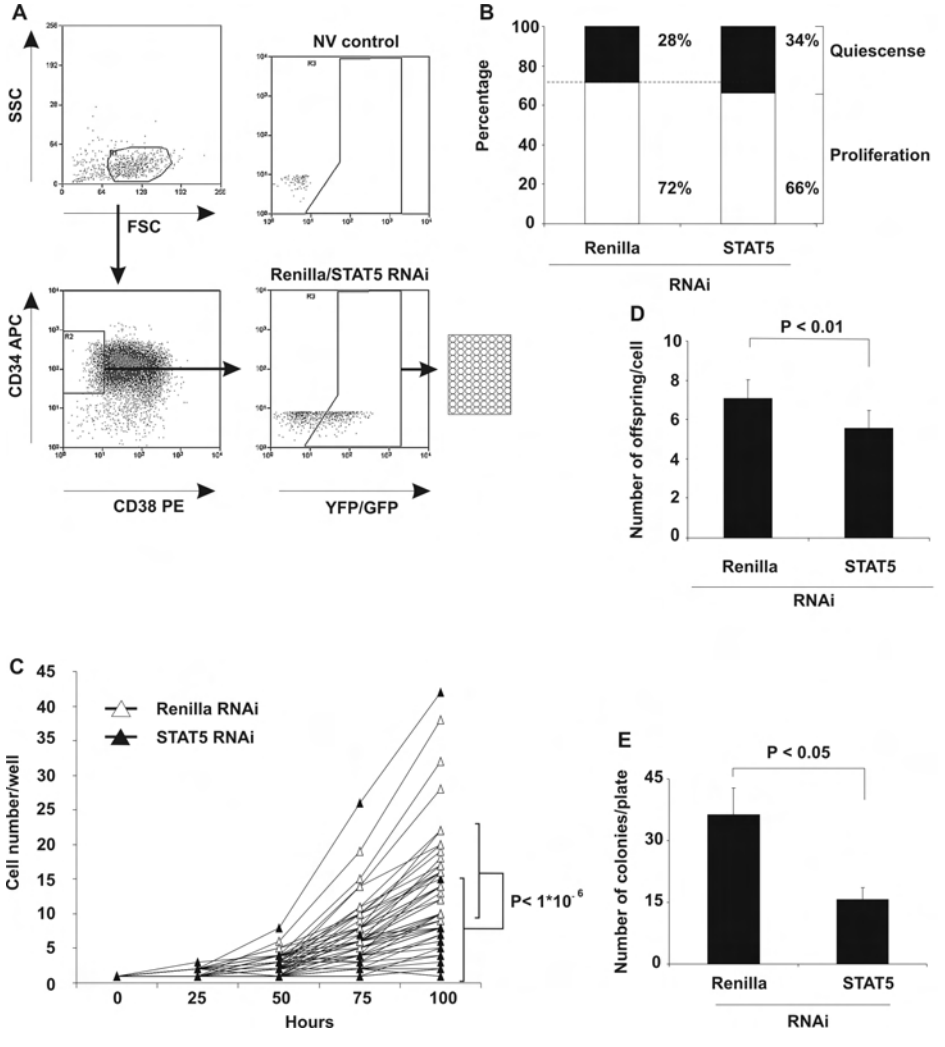

Figure 3 STAT5 knockdown impairs outgrowth of primitive CD34$^+$ CD38$^-$ cells.
(A) Sorting scheme for obtaining single CD34$^+$ CD38$^-$ YFP$^+$ (Renilla) or GFP$^+$ (STAT5 RNAi) cells in 96-wells format. NV indicates a non-transduced (No Virus) control to set gates. (B) Percentage of wells quiescent or proliferating after STAT5 knockdown. Indicated percentages are averaged from 3 independent experiments. (C) Cell number per proliferating well at the indicated time points after sorting. 30 individual wells per group are shown. Bars indicate average cell number with standard deviations at t = 100 hrs. A student t-test was performed to calculate statistical differences between the two groups. N=3. (D) The average number of daughter cells (offspring) produced per proliferating cell/well. A student t-test was performed to calculate statistical differences between the two groups. Error bars denote standard deviations, N=3. (E) The average number of CFC-colonies produced by proliferating cells per plate. A student t-test was performed to calculate statistical differences between the two groups. Error bars denote standard deviations, N=3.

Activated STAT5 is frequently observed in AML cells, which is efficiently downregulated after lentiviral-mediated RNAi

STAT5 knockdown affected the proliferation rate of $CD34^+/CD38^-$ cells. For malignant AML cells, this primitive cell compartment was exclusively shown to contain SCID Leukemia-Initiating Cells.[32] Since constitutive phosphorylation and activation of STAT5 has been described for AML patients[410] we were interested in the effect of STAT5 knockdown in self-renewing leukemic AML stem/progenitor cells. The AML's were classified, according to FAB-classification, as M1 (n=3), M2 (n=4), M4 (n=3) and M5 (n=5). PCR studies demonstrated that 7 out of 15 AML's had FLT3-ITD mutations. Figure 4A depicts a western blot analysis of 8 primary AML samples and demonstrates the presence of phospho-STAT5 in 5 patients (62.5%), thus confirming previously published results.[40;234] Subsequently, the subpopulation of $CD34^+$ AML cells (n=11) were sorted and transduced with our lentiviral vectors. Flow cytometric analysis demonstrated comparable transduction efficiencies of AML $CD34^+$ cells compared to CB $CD34^+$ cells, ranging between 50% and 95% (fig. 4B and 1B). Western blot analysis confirmed efficient downregulation of STAT5 in these AML $CD34^+$ cells (fig. 4C), which varied from 20 to 85% as being normalized against STAT3 or Actin protein.

Knock-down of STAT5 impairs long-term growth of leukemic cells on MS-5 co-cultures

To evaluate the effects of STAT5 knock-down on the proliferation of AML $CD34^+$ cells, transduced cells were plated onto MS-5 stromal cells and subsequently followed for growth and expansion (N=11). Cultures of AMLs with FAB classification M2 failed to expand and were terminated. Figure 4D demonstrates 4 representative cumulative growth cultures of the remaining cultures. All expanding $CD34^+$ AML cultures transduced with the STAT5 hairpin (N=8) demonstrated an impaired growth compared to control transduced cultures, although the level of reduction in growth was variable. The average reduction in cell number after STAT5 RNAi compared to control cells was 72% ± 17%, $P< 1*10^{-5}$. This reduction was also observed, when relative percentages of YFP^+ (Renilla control) and GFP^+ (STAT5 hairpin) cells were calculated in expanding AML cultures. GFP percentages indicated a decline similar to the decline in CB $CD34^+$ MS-5 co-cultures (fig. 4E, data shown is an average of 7 AML cultures, compare to figure 1F).

Furthermore, the expansion of these AML cultures was calculated. Figure 4F depicts that STAT5 knockdown resulted in an almost 5-fold decreased expansion from an average 80-fold expansion (+/- 58) of control transduced cultures at week 6, to an average 17-fold expansion (+/- 15) for STAT5 hairpin

transduced cultures (P<0.05). Together these data suggest that CD34^{+} AML cells are also dependent upon STAT5 signaling for their long-term growth.

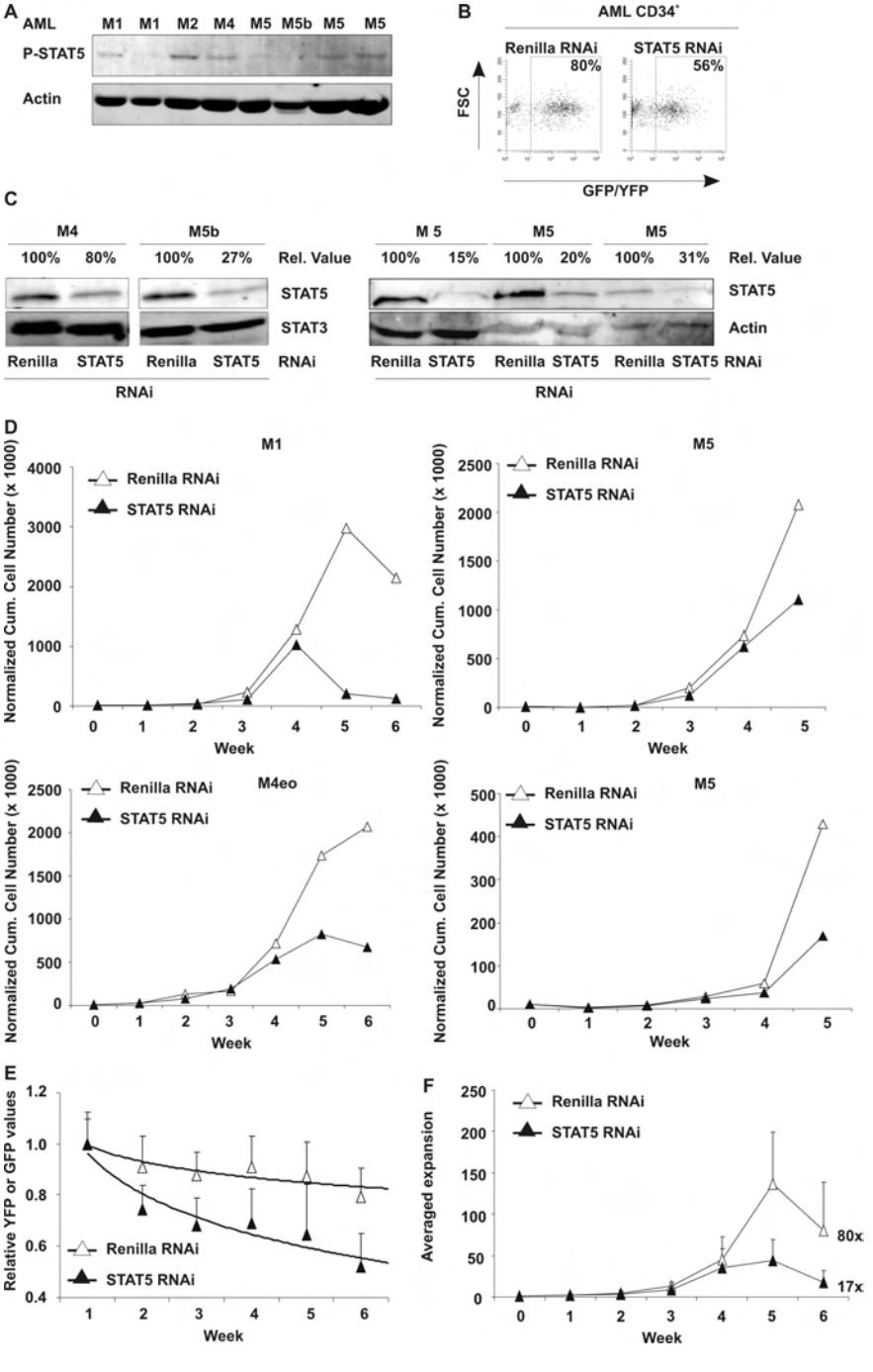

Figure 4 Efficient STAT5 knockdown in CD34$^+$ AML cells impairs long-term growth on MS5 co-culture.
(A) Western blot analysis for Phospho-STAT5 in 8 AML patients. Actin is shown as loading control. (B) Transduction efficiency of CD34$^+$ AML cells, performed as indicated in Material and methods (M&M) (C) Western blot analysis of STAT5 levels in control (Renilla) or STAT5 RNAi transduced CD34$^+$ cells. Percentages indicate expression of STAT5 relative to control cells, normalized against STAT3 or Actin levels. Quantitative western blot analysis was performed on an Odyssey infrared scanner or using Quantity One imaging software from BioRad. (D) Cumulative cell counts of control (Renilla) or STAT5 RNAi transduced CD34$^+$ AML cells on MS5 co-culture. All cell counts were normalized against week 0, the time of plating. 4 representative cultures are shown. N=11. (E) Transduced CD34$^+$ AML cells in long-term co-culture on MS5 stromal cells. Shown are average YFP (Renilla control) or GFP STAT5 RNAi percentages relative to week 1. A trendline is indicated and error bars denote standard deviations. N = 7. (F) Averaged calculated expansion of 7 AML MS5 co-cultures, with error bars indicating standard deviations. The average expansion at week 6 is indicated next two the experimental group.

Discussion

The present study demonstrates that limited reduction of STAT5 expression especially affects the hematopoietic stem/progenitor pool. In our MS5 co-culture experiments we observed a growth reduction, without affecting the differentiation program of the myeloid and erythroid lineages. This finding is remarkable since a wide variety of hematopoietic growth factors and cytokines, including FLT3-L, SCF, G-CSF, GM-CSF, IL-3, EPO and TPO, have been shown to induce STAT5 transactivation and affect the differentiation of human stem/progenitor cells.[218-224] These differences in cell biological effects downstream of STAT5 most likely find its origin in differences in cell type, (combinations of) cytokine signals and concentrations of cytokines (reviewed in[225;226]). Our observations regarding differentiation correspond to the relatively mild phenotype of STAT5AB$^{-/-}$ mice. In steady-state conditions these mice displayed normal levels of erythrocytes, platelets, neutrophils and monocytes in the peripheral blood [239]

In contrast, distinct phenotypes were observed by downmodulation of STAT5 expression in the HSC and progenitor subpopulations, since a significant reduction in progenitor and LTC-IC frequencies was observed. Recently, Scherr et al. have reported that normal human CD34$^+$ progenitors were less sensitive to STAT5 RNAi then CML CD34$^+$ progenitors,[411] but their reductions of ~45% for normal CD34$^+$ progenitors are comparable to our findings. Furthermore, their and our observed reduction in colony numbers after STAT5 RNAi corresponded with the reductions seen in CFU-mix colonies (~43%) and in CFU-GM colonies (~53%) in STAT5AB$^{-/-}$ mice.[239] These findings suggest that the reduction in STAT5 levels, as obtained by RNA interference, is sufficient to induce similar hematopoietic phenotypes as the

100% STAT5 reduction in the STAT5AB knock-out mice, suggesting that our observations are not due to differences in transduction rates or RNAi efficiency.

Both progenitors and more primitive cells, determined with LTC-IC assays, displayed reduced frequencies in STAT5A RNAi transduced cells. In addition, single cell experiments with $CD34^+CD38^-$ cells demonstrated that the number of daughter cells produced by STAT5 RNAi cells is lower than in control cells and gives rise to fewer colonies in methylcellulose. Therefore, we speculate that a more primitive cell, e.g. a common myeloid progenitor (CMP) or a HSC is being affected by STAT5 reduction. Interestingly, HSCs from STAT5AB null mice with impaired repopulation potential, displayed a reduction in spleen colony forming units.[240;241] Those CFU-Ss were smaller than those from wild-type cells, indicating fewer progeny cells produced per colony, which is in line with our findings. Whether STAT5 affects both primitive progenitors (CMP) and HSCs or only HSCs, with subsequent reductions in downstream progenitors needs further elucidation by means of prospective purification steps of STAT5 RNAi-transduced HSCs versus CMPs.

How STAT5 affects these stem/progenitor cells remains unclear. In $STAT5AB^{-/-}$ mice increased apoptosis in erythroid progenitors has been assigned to decreased transcription of the Bcl-X_L gene[405;406] and restoration of Bcl-X_L could fully compensate for this effect.[412] In our co-cultures, however, we did not detect an increased rate of apoptosis and also STAT5 RNAi-transduced $CD34^+$ CB cells did not display a decreased expression of the Bcl-X_L gene (preliminary data, not shown). On the other hand, preliminary data on Cyclin D1 gene expression demonstrated a reduction in STAT5 RNAi-transduced $CD34^+$ CB cells (data not shown), which is consistent with a defect in proliferation of bone marrow-derived macrophages of STAT5A-deficient mice.[413] Further studies in purified $CD34^+CD38^-$ cells transduced with STAT5 RNAi should provide more insight in the processes of apoptosis and proliferation.

Overexpression of a constitutively active STAT5 enhanced the self renewal potential of $CD34^+$ cells.[36;37] In mice, this was coupled to the development of myeloproliferative disease (MPD) or multilineage leukemias.[36;414] Detailed analysis of derivatives of this constitutive active STAT5 showed that the enhanced self-renewal or MPD/multilineage leukemia was potentially due to enhanced tetramer formation and hence more stable DNA binding of constitutive active STAT5 mutants to promoters of target genes.[414] Interestingly, similar STAT5 tetramers were observed in various AML, chronic myeloid leukemia (CML) and acute lymphoid leukemia (ALL) patient samples.[414] In addition, STAT5 activation has been shown to be critical for BCR-ABL dependent transformation of hematopoietic cells.[411;415-418]

For AML, constitutive activation of STAT5 has been demonstrated,[40;234] but the role of STAT5 signaling in the pathogenesis of AML remained so far elusive. This is predominantly hampered by experimental limitations. Gene transfer into primary AML have shown stable targeting efficiencies,[419;420] but in liquid culture these cells lost their viability after approximately 15 days. Although AML SCID-Leukemia Initiating Cells (AML SL-IC) could sustain longer growth in (NOD)-SCID mice[32;421] this assay was sofar not combined with molecular interference in signal transduction pathways. Furthermore, it was demonstrated that the in vivo NOD-SCID model also has limitations, as almost half of the investigated AML cases did not engraft in irradiated mice.[422] Therefore we used an AML culture assay in which expansion of transduced leukemic cells could be followed for prolonged periods of time.

The results indicate that by lentiviral transduction RNA interference allows us to study the importance of STAT5 activation in sorted $CD34^+$AML cells. Knockdown of STAT5 in AML $CD34^+$ cells shows a reduction in cumulative cell numbers within 5 to 6 weeks of co-culture on MS5. This reduction in expansion of AML cells upon STAT5 RNAi was similar to the decline we observed in $CD34^+$ CB cells, suggesting that a comparable cell type, either a primitive progenitor or a stem cell is being affected. In addition no changes for myeloid and erythroid markers between STAT5 RNAi and control AML cultures were observed (data not shown), in line with the results of CB $CD34^+$ cells.

Our data indicate that the concomitant reduction in STAT5 phosphorylation in the STAT5 RNAi transduced cells is sufficient to limit the expansion of the leukemic stem/progenitor pool. From a clinical point of view this is an important observation, since a partial reduction of STAT5 already gives a distinct phenotype. This would imply that alternative strategies e.g. blocking kinase activity, which result in a partial reduction in STAT5 activity, will have distinct effects on the leukemic stem cell compartment. Indeed, a phase 1 clinical study with SU11248, a multitarget kinase inhibitor against Flt3, PDGFR, VEGFR and KIT, demonstrated marked reductions (>50%) in STAT5 phosphorylation in peripheral blood (PB) of AML patients and a decreased blast count in some of those.[423] Although not all patients in that study showed significant changes in pheripheral blood blast counts after 48 hours, our in vitro culture assay demonstrates that it may require several weeks before a distinct effect can be observed.

In conclusion, our data indicate that STAT5 expression is required for the maintenance and expansion of both normal as well as leukemic stem/progenitor cells.

Acknowledgements

The authors would like to acknowledge Geert Mesander and Henk Moes for help with Flow Cytometry; Prof. Dr. H. Spits from the Department of Cell Biology and Histology, Amsterdam Medical Center, Division of Immunology, Netherlands Cancer Institute, Amsterdam for the kind gifts of pTRIP Renilla RNAi and pTRIP STAT5 RNAi; and Amgen Inc. and Kirin for providing cytokines. The authors greatly appreciate the help of Dr. A. van Loon, Dr. J. J. Erwich and colleagues (Obstetrics departments from the Martini Hospital and UMCG) for collecting cord blood.

Re-introduction of C/EBPα in leukemic CD34^{+} stem/progenitor cells impairs self-renewal and partially restores myelopoiesis

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Chapter 8

Abstract

The CCAAT/enhancer binding protein (C/EBP) α transcription factor is indispensable for myeloid differentiation. In various myeloid leukemias, C/EBPα is mutated or functionally impaired due to decreased C/EBPα expression or phosphorylation. In order to investigate the functional consequences of decreased C/EBPα function in AML, we re-introduced C/EBPα in primary $CD34^+$ sorted AML cells using a lentiviral approach. Self-renewal and differentiation of primary AML stem cells were studied on long-term MS5-cocultures. Activation of C/EBPα immediately led to a growth arrest in all AML cultures (N=7), resulting in severely reduced expansion as compared to control cultures. This growth arrest corresponded with enhanced myeloid differentiation as assessed by FACS analysis for CD14, CD15 and CD11b. Myeloid differentiation was further confirmed by the upregulation of Neutrophil Elastase and G-CSF-Receptor in C/EBPα transduced cells. C/EBPα-expressing AML $CD34^+$ cells failed to generate 2^{nd} and 3^{rd} leukemic cobblestone areas (L-CAs) in serial replating experiments, while control cultures could be sequentially passaged for over 4 times, indicating that re-introduction of C/EBPα impaired the self-renewal capacity of the leukemic $CD34^+$ compartment. Together, our data indicate that low C/EBPα levels are necessary to maintain self-renewal and the immature character of AML stem cells.

Introduction

The transcription factor C/EBPα is a major determinant of myeloid differentiation. Its expression is low in stem cells and uncommitted progenitors and is upregulated when common myeloid progenitors (CMP) and granulocyte-macrophage progenitors (GMP) develop.[8;246] Concomitantly, the expression ceases with the arrival of the megakaryocytic-erythroid progenitor and is abolished in lymphoid cells.[8] Overexpression in cells with GMP potential indicated that C/EBPα induces granulocytic differentiation and blocks the monocytic differentiation program.[246] This is in line with the granulocytopenia in C/EBPα$^{-/-}$ mice, without affecting other lineages.[245] Although initial studies did not show effects on monocyte/macrophage development, conditional knock-out mice for C/EBPα have shown that this development is indeed affected as well.[424] Recently, overexpression studies indicated that C/EBPα could also direct monocytic development,[247] which is consistent with data demonstrating a requirement for C/EBPα in the transition from CMP to GMP,[253] but not for development towards mature cells.

Furthermore, C/EBPα has been implicated in the regulation of HSC self-renewal. The repopulating ability of C/EBPα$^{-/-}$ HSCs is increased compared to wildtype (wt) HSCs.[253] Also, these mice demonstrate an accumulation of immature myeloid blasts in the bone marrow upon transplantation with C/EBPα$^{-/-}$ HSCs.[253] The block in myeloid differentiation combined with an enhanced self-renewal, closely resembled some characteristics of leukemic development. Indeed, C/EBPα was suggested to be a common denominator in human AML.[85] In the last few years various mechanisms have been reported, through which C/EBPα is negatively regulated in various AML subtypes. Mutations in the N-terminus have been described in patients with a FAB M1 and M2 subtype without AML-ETO translocation.[81;254;255] These N-terminal mutations give rise to a truncated dominant negative form of C/EBPα , which interferes with DNA-binding and transactivation of the wt C/EBPα.[254] C-terminal mutations of C/EBPα have been described in FAB subtypes M1, M2 and occasionally in M4 and result in deficiency in DNA binding of C/EBPα.[256;257] AMLs with an AML-ETO translocation have been demonstrated to downregulate C/EBPα at the transcriptional level,[81] although some studies have also shown that AML-ETO can associate with and inhibit C/EBPα function.[258] In AML patients with Flt3-ITD mutations or inv16, suppression of C/EBPα expression was demonstrated,[259;425] whereas functional inhibition of C/EBPα through Flt3-ITD-induced phosphorylation has also been reported.[260] Promoter hypermethylation might also reduce C/EBPα expression in at least some AMLs[426] and lastly, the

fusion gene AML1-MDS1-EVI1 has been demonstrated to suppress DNA binding of C/EBPα.[261] Together, all these mechanisms lead to decreased functionality of C/EBPα in terms of DNA binding and the expression of target genes, such as G-CSF Receptor, M-CSF-Receptor and the IL-6 Receptor.[245;424;427] This block at the CMP stage in the differentiation program, combined with the enhanced self-renewal potential, may subsequently contribute to leukemic transformation.

Overexpression studies of C/EBPα in the leukemic AML-ETO expressing Kasumi-1 cell-line and in Flt3-ITD expressing 32D and MV4;11 cell-lines have demonstrated that impaired myeloid differentiation could be rescued.[81;259;260] Recently, we demonstrated that the STAT5-induced erythroid differentiation and the subsequent block in myeloid differentiation of human Cord Blood (CB) $CD34^+$ cells involves downmodulation of C/EBPα,[37] which could be counterbalanced by re-introduction of C/EBPα.[244] In studies in which AML patients were treated with a Flt3 inhibitor CEP-701, re-expression of C/EBPα was observed after 4 weeks of treatment and this was correlated with an enhanced expression of surface markers CD11b and CD15.[259] However, a direct relation between these two events was not yet established in primary AML samples. This prompted us to re-introduce C/EBPα into AML blasts. For these studies we used the $CD34^+$ sorted subfraction from the blast cell population. This cell fraction contains the AML Long Term Culture-Initiating Cell (AML-LTC-IC)[102] activity and might be most informative regarding the cellular functions of C/EBPα in long-term leukemic stem cell cultures. The results demonstrate that re-introduction of C/EBPα in primary $CD34^+$ sorted AML cells leads to a rapid growth arrest, with an associated increased expression of differentiation markers. This enhanced differentiation is accompanied by a loss of self-renewal, as assessed by serial replating of leukemic stem cells. We thus conclude that downregulation of C/EBPα function in primary human AMLs is necessary for maintenance of the leukemic phenotype by keeping them undifferentiated and uphold their self-renewal.

Materials and methods

Long-term cultures on stroma - AML blasts from peripheral blood or bone marrow from untreated patients with AML were studied after informed consent. The protocol was approved by the Medical Ethical Committee. AML mononuclear cells were isolated by density gradient centrifugation and $CD34^+$ cells were selected by MiniMACS (Myltenyi Biotec) or sorted on a MoFlo

(DakoCytomation, Carpinteria, CA, USA). After transduction, 200.000 cells were plated onto T25 flasks pre-plated with MS5 stromal cells and expanded in Long Term Culture (LTC) medium (αMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% horse serum (Sigma, Zwijndrecht, The Netherlands) penicillin and streptomycin, 200 mM Glutamine, 57.2 µM β-mercaptoethanol (Sigma) and 1 µM hydrocortisone (Sigma)) supplemented with 20 ng/ml Interleukin 3 (IL-3; Gist-Brocades, Delft, The Netherlands), Granulocyte Colony Stimulating Factor (G-CSF; Rhone-Poulenc Rorer, Amstelveen, The Netherlands) and Thrombopoietin (TPO; Kirin, Japan). Cultures were kept in the presence or absence of 500 nM 4-hydroxy tamoxifen (4-OHT) at 37ºC and 5% CO_2. Cultures were demidepopulated weekly for analysis. Cord blood (CB) $CD34^+$ cells were derived from neonatal cord blood from healthy full-term pregnancies after informed consent from the Obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands and isolated by MiniMACS selection.

Flow cytometry analysis - All fluorescence activated cell sorter (FACS) analyses were performed on a FACScalibur (Becton Dickinson (BD), Alpen a/d Rijn, The Netherlands) and data was analyzed using WinList 3D (Topsham, USA). Cells were sorted on a MoFlo. Antibodies were obtained from BD.

Lentiviral transductions - $2.5x10^6$ 293T Human Embryonic Kidney cells were transduced with 3 µg pCMV Δ8.91, 0.7 µg VSV-G and 3 µg of pRRL-Venus or pRRL-C/EBPα-ER (pRRL-Venus was a kind gift from Prof. Dr. C. Baum). pRRL-C/EBPα-ER was cloned by swapping the EcoRI fragment from C/EBPα-ER from MiNR1-C/EBPα-ER (described previously)[244] blunt into the SnaB1 site of pRRL-Venus. After 24 hours medium was changed to HPGM (Cambrex, Verviers, Belgium) and after 12 hours supernatant containing lentiviral particles was harvested and stored at -80ºC. $CD34^+$ AML blasts and CB $CD34^+$ cells were isolated with MiniMACS columns and subsequently cultured in RPMI supplemented with 10% FCS, 20 ng/ml IL-3, G-CSF and TPO for 4 hours or in HPGM supplemented with c-Kit ligand), Flt-3 ligand (both from Amgen, USA) and TPO (100 ng/ml each) for 16 hours at 37ºC and 5% CO_2 respectively. AML blasts were transduced in 3 consecutive rounds of 8 to 12 hours with lentiviral supernatant supplemented with 10% FCS, 20 ng/ml IL-3, G-CSF and TPO, and Polybrene (4 µg/ml; Sigma) and CB $CD34^+$ cells in 2 consecutive rounds of 8 to 12 hours with lentiviral supernatant supplemented with c-Kit Ligand/Flt-3 ligand/TPO (100 ng/ml each) and Polybrene (4 µg/ml). Transduction efficiency was measured by FACS analysis and overexpression was investigated by means of Western blot using antibodies against C/EBPα (N19, Santa Cruz, CA,

USA) and GFP (recognizes Venus as well, Molecular probes, Invitrogen, Breda, The Netherlands) in a 1:1000 dilution in PBS on an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA) or using ECL according to the manufacturer's instructions (Roche Diagnostics, Almere, The Netherlands).

(*Quantitative) PCR* - gene expression for GCSF-Receptor, neutrophil elastase (NE) and C/EBPα was investigated by means of (quantative) RT-PCR (Q-PCR), sequences and conditions are available on request). For RT-PCR, total RNA was isolated from 1 x 10^5 to 1 x 10^6 cells using the RNeasy kit from Qiagen (Venlo, The Netherlands) according to the manufacturer's recommendations. RNA was reverse transcribed with M-MuLV reverse transcriptase (Roche Diagnostics). For PCR, 2 µl of cDNA was amplified (sequences and conditions are available on request) in a total volume of 25 µl using 2 units of Taq polymerase (Roche Diagnostics). As a negative control RNA minus reverse transcriptase (-RT) prepared cDNA was used in PCR reactions. 10 µl aliquots were run on 1.5% agarose gels. For real-time RT-PCR, 2 µl aliquots of cDNA were real-time amplified using iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands) on a MyIQ thermocycler (Bio-Rad) and quantified using MyIQ software. GAPDH expression was used to calculate and normalize expression of all genes investigated.

Electrophorectic Mobility Shift Assays (EMSAs) - Nuclear extracts of 25.000 sorted $CD34^+$ (transduced) AML cells were prepared according to the mini-extracts method.[273] EMSA analysis was performed by incubating 5 µl of nuclear extract with 5'-IRDye 700 labelled double stranded oligonucleotides for 30 minutes at room temperature. Binding reactions were run on non-denaturing 4 % Acrylamide gels in 1 x TBE and the gels were scanned using an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA) To check for specificity of the reactions, 50 fold molar excess of unlabeled oligonucleotide (either self or non-self) was added to the binding reactions. For supershift analysis, 1 µl of C/EBPα specific antibody (SC-61, Santa Cruz Biotech, Santa Cruz, CA, USA) was added simultaneously with the probe.

Microscopy and cytospins - For morphological analysis, May-Grünwald Giemsa (MGG) staining was used to analyze cytospins. Pictures were taken on an Olympus BX50 microscope (Olympus Nederland BV, Zoeterwoude, The Netherlands) with a 100x/1.3 oil objective. Pictures of MS5 cocultures and ($Venus^+$) L-CAs were taken with a Leica DM-IL microscope (Leica Microsystems, Rijswijk, The Netherlands) with a 20x/0.30 or 40x/0.60 objective.

Replating of leukemic cultures – For assessment of self renewal, 200.000 AML cells were transduced and plated onto T25 flasks pre-plated with MS5 stromal cells. AML cells were expanded in LTC medium supplemented with 20 ng/ml IL-3, G-CSF, TPO and 500 nM 4-hydroxy tamoxifen (4-OHT). At week 1, 3 and 5 of culture, fresh medium was added. At week 2, 4 and 6 all medium containing suspension cells was removed and analysed by flow cytometry for the percentage of Venus-expressing cells. The remaining adherent cell layer, containing both MS5 cells and leukemic cobblestone area forming cells (LCA), was washed twice with PBS and trypsinised for 15 minutes. All trypsinised cells were resuspended in 5 ml LTC medium and 10% was replated onto T25 flasks pre-plated with fresh MS5 stromal cells. The remaining 90% was stained with PE-labelled anti-human CD45 (BD) to distinguish between mouse MS5 and human leukemic cells and analyzed for Venus expression.

Results

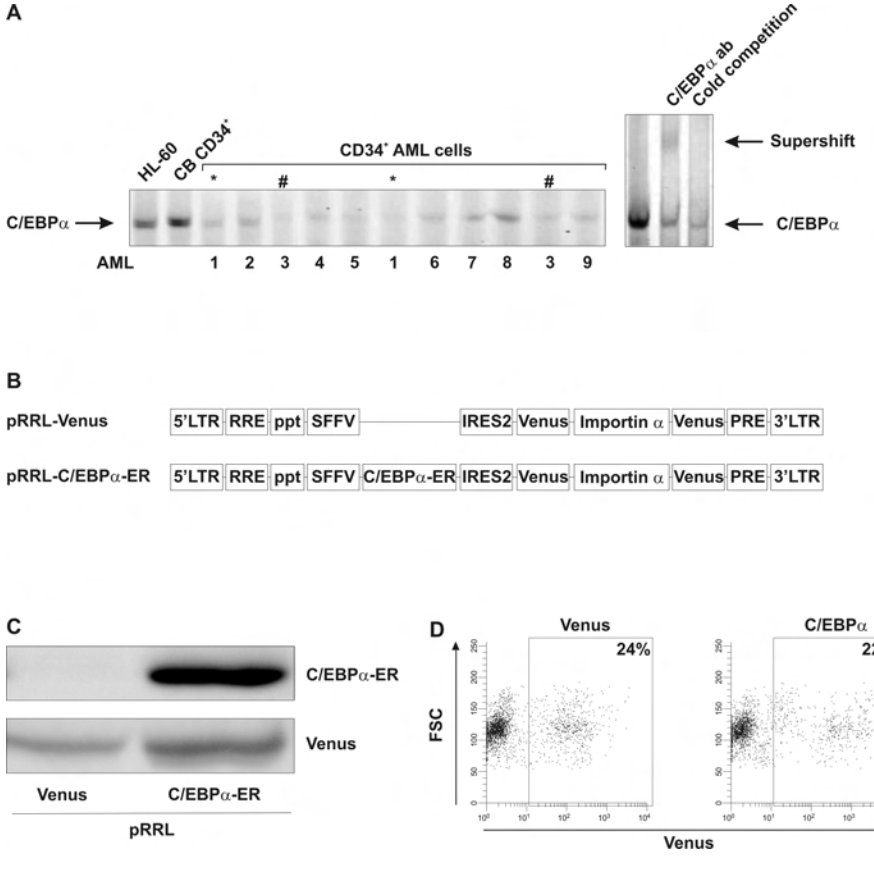
Impaired DNA-binding capacity of C/EBPα in CD34$^+$ AML cells

In order to study C/EBPα DNA binding in the primitive subfraction of AML cells, CD34$^+$ AML cells, which have been demonstrated to posses AML Long Term Culture-Initiating Cell (AML-LTC-IC) and Leukemic Cobblestone Areas Forming Cell (L-CAFC) activity[102;428] (and van Gosliga et al., submitted manuscript) from FAB subtypes M1 (N=3), M2 (N=2), M4 (N=1) and M5 (N=5) were isolated and subjected to nuclear fractionation and gel shift (EMSA) experiments. 7 out of 11 AMLs tested were Flt3-ITD positive (63.6%, table 1). Figure 1A demonstrates that DNA binding of C/EBPα in various AML samples is severely reduced as compared to C/EBPα DNA binding from extracts of CB CD34$^+$ cells and the myeloid HL-60 cell-line. Sorting of equal cell numbers was used to normalize protein content of the extracts. The gel-shift analysis was specific for C/EBPα, since competition with an excess of unlabeled probe demonstrated a decrease in DNA binding activity (fig. 1A, box on the right, cold competition). Furthermore, addition of a C/EBPα specific antibody resulted in a supershift, demonstrating the presence of C/EBPα in the protein-DNA probe complex (fig. 1A, super shift). These data show that the transcription factor C/EBPα has a reduced DNA binding capacity in the majority of AML cases studied.

Table 1 Summary of patient characteristics.

						Experiment	
Patient No	BM/PB	Flt3 ITD	FAB	karyotype	Risk Group	EMSA	C/EBPα transduction
1	PB	-	M2	t(8;21)	good	+	
2	PB	+	M2	t(11;17)?	poor	+	+
3	PB	+	M5	normal	intermediate	+	+
4	PB	-	M1	+3q, -7, -10	poor	+	
5	PB	-	M1	normal	intermediate	+	
6	PB	-	M4	t(6;11), (q27;23)	poor	+	
7	PB	+	M5	normal	intermediate	+	+
8	PB	+	M5b	normal	intermediate	+	+
9	PB	+	M5	normal	intermediate	+	+
10	PB	+	M5	normal	intermediate	+	+
11	PB	+	M1	normal	intermediate	+	+

Blasts isolated from bone marrow (BM) or peripheral blood (PB). AMLs were categorized according to the French-American-British (FAB) classification. Positivity for Flt3-ITD is indicated as well as karyotype and riskgroup. The right part indicates the experiments performed with the AMLs.



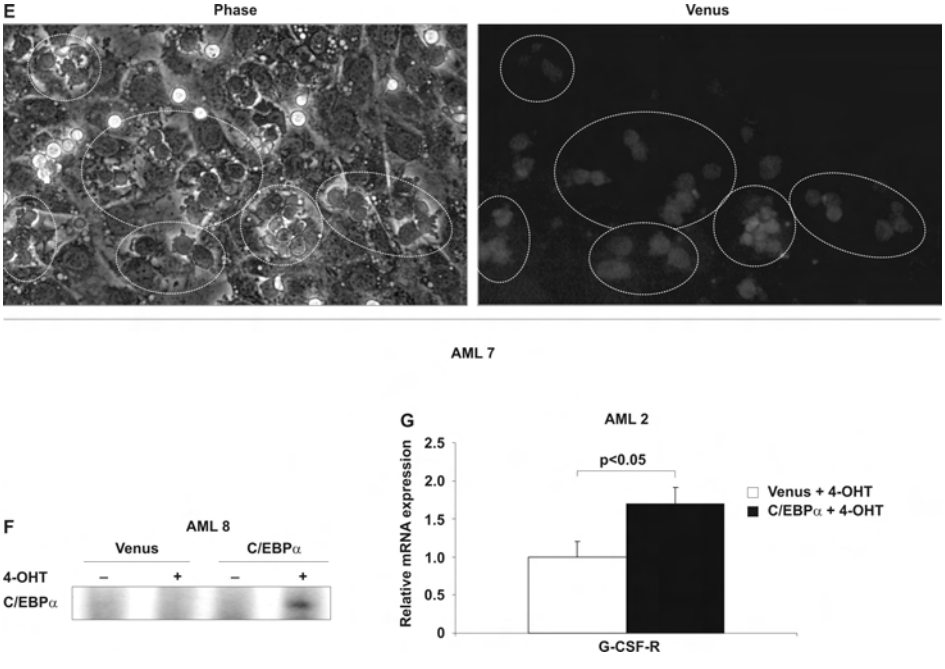


Figure 1 C/EBPα has impaired DNA-binding capacity in AML patients and can be restored upon transduction into primitive CD34$^+$ AML cells.
(A) DNA-binding experiment to C/EBPα specific probe with nuclear extracts of 25.000 sorted CD34$^+$ AML cells. Nuclear extracts of 25.000 HL-60 and CB CD34$^+$ cells are shown as control. Nuclear extracts taken from the same patients, but harvested in independent experiments (marked * and #) indicate that decreased C/EBPα DNA binding is not due to the experimental set up or experimental variations. The box on the right demonstrates C/EBPα-specific controls on nuclear extracts from 33.000 HL-60 cells: A supershift with a C/EBPα-specific ab and a cold competition with a 50-fold excess of unlabelled probe.
(B) Schematic overview of the lentiviral overexpression vectors used in these studies. (C) Western blot analysis of C/EBPα-ER in CD34$^+$ CB stem/progenitor cells. (D) Transduction efficiency of CD34$^+$ AML cells. (E) Phase contrast and Venus fluorescence (GFP channel) pictures indicating the presence of Venus$^+$ cobblestone areas (CA) (encircled) in week 2 leukemic MS5 cocultures. (F) DNA-binding experiment to C/EBPα specific probe with nuclear extracts of 50.000 CD34$^+$ AML cells from MS5 cocultures treated for a week with or without 500 nM 4-hydroxy tamoxifen (4-OHT). (G) Q-PCR for the C/EBPα target gene G-CSF-Receptor (G-CSF-R) after 1 week of coculture on MS5 in the presence of 500 nM 4- OHT. mRNA levels were normalized against GAPDH mRNA levels and C/EBPα-transduced values are shown relative to control Venus-transduced values. An average of two samples is shown with SEM.

Efficient transduction of CD34$^+$ AML cells using lentiviral vectors containing an inducible form of C/EBPα

In order to investigate the consequences of the reduced C/EBPα DNA binding, we employed lentiviral vectors in order to re-introduce C/EBPα into human CD34$^+$ AML cells (schematically depicted in fig. 1B). C/EBPα was fused to the estrogen receptor ligand binding domain (ER) to create a 4-hydrotamoxifen (4-OHT) inducible form of C/EBPα, which was inserted in front of the IRES2

sequence. These vectors furthermore contained a Venus (an optimized YFP variant) cDNA fused to importin α sequences to target it to the nuclear envelope. Western blot analysis of CB CD34$^+$ transduced cells demonstrated high expression from the Spleen Focus Forming Virus (SFFV) promoter (fig. 1C).

Primary CD34$^+$ sorted AML cells were transduced with efficiencies between 20 and 25% (fig. 1D). Fluorescence microscopy on MS5 co-cultures showed the presence of Venus positive leukemic Cobblestone Areas (L-CA) (fig. 1E). As these L-CAs contained secondary and tertiary plating efficiency these data indicate the technical ability to transduce an immature CD34$^+$ cell fraction that contained self-renewal capacity (fig. 3A and data not shown). Venus positive AML cells were sorted, treated with 4-OHT and nuclear extracts were subjected to gel-shift analysis in order to verify the validity of our experimental setup. Figure 1F demonstrates DNA binding of exogenous C/EBPα-ER upon administration of 4-OHT. Furthermore, a Q-PCR analysis for the C/EBPα target gene G-CSF-Receptor (G-CSF-R) was performed on transduced AML cells, to verify that the 4-OHT-induced DNA binding is also functional. As is depicted in figure 1G, activation of C/EBPα-ER with 4-OHT induced an 1.7-fold upregulation of G-CSF-R expression ($p < 0.05$) in primary CD34$^+$ AML cells, indicating that C/EBPα is functionally active in our transduced leukemic cells.

Table 2 Expansion and surface marker expression after transduction.

		Fold expansion	% CD marker in culture					Increase or decrease in CD marker expression				
AML #			CD14	CD15	CD71	CD11b	CD36	CD14	CD15	CD71	CD11b	CD36
2	Venus + 4OHT	3.9	0%	0%	nd	nd	nd					
	C/EBPα + 4OHT	0.4	20%	16%	nd	nd	nd	20% ↑	16% ↑	nd	nd	nd
3	Venus + 4OHT	28,0	15%	43%	22%	nd	2%					
	C/EBPα + 4OHT	2.2	88%	84%	15%	nd	21%	73% ↑	41% ↑	-7% ↓	nd	19% ↑
7	Venus + 4OHT	87.5	38%	41%	37%	nd	7%					
	C/EBPα + 4OHT	5.7	69%	36%	14%	nd	9%	31% ↑	-5% ↓	-23% ↓	nd	2% ↑
8	Venus + 4OHT	863.5	3%	1%	55%	66%	6%					
	C/EBPα + 4OHT	39.6	43%	8%	50%	91%	20%	40% ↑	7% ↑	-5% ↓	25% ↑	14% ↑
9	Venus + 4OHT	4.5	12%	42%	23%	nd	7%					
	C/EBPα + 4OHT	0.4	22%	76%	10%	nd	4%	10% ↑	34% ↑	-13% ↓	nd	-3% ↓
10	Venus + 4OHT	16.3	15%	0%	27%	63%	3%					
	C/EBPα + 4OHT	1.5	31%	11%	20%	92%	10%	16% ↑	11% ↑	-7% ↓	29% ↑	7% ↑
11	Venus + 4OHT	154.5	1%	2%	60%	65%	2%					
	C/EBPα + 4OHT	2,0	3%	0%	6%	89%	1%	2% ↑	-2% ↓	-54% ↓	24% ↑	-1% ↓

The calculated fold-expansion at week 4, as well as the percentages of cells positive for cell surface markers as measured by flow cytometry are indicated. The arrows indicate the difference in expression between control Venus-transduced cells treated with 500 nM 4-OHT and C/EBPα-ER-transduced cells after 1 week of coculture on MS5 cells. nd = not determined.

Restoration of C/EBPα expression in CD34$^+$ sorted AML blasts induces a cell-cycle arrest, in conjunction with enhanced expression of CD14, CD15 and CD11b

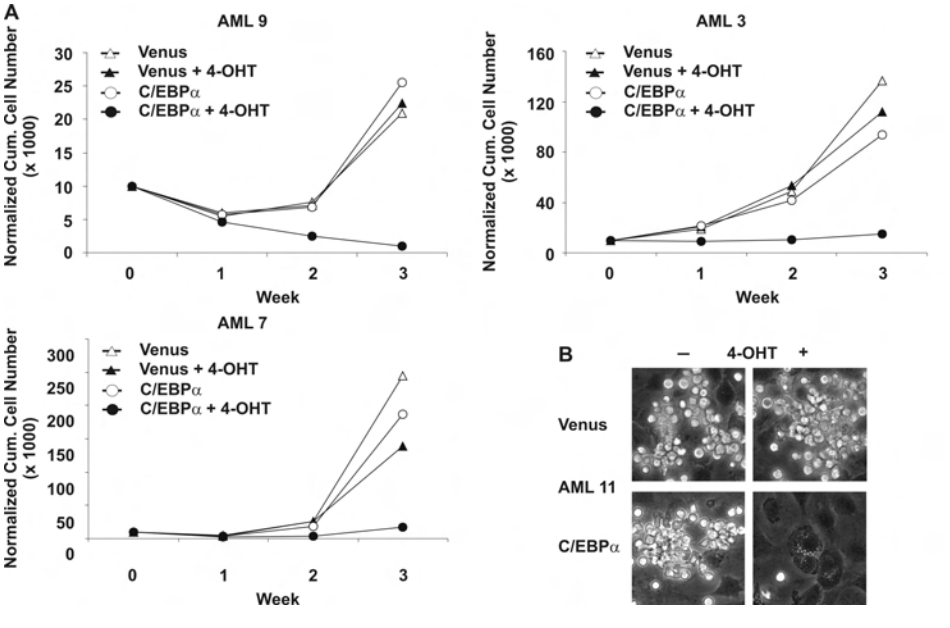
Subsequent C/EBPα expression was restored by transduction of AML CD34$^+$ cells, followed by plating onto MS-5 stromal cells in order to study proliferation, differentiation and expansion (N=7). Figure 2A demonstrates 3 independent cumulative growth cultures. Control cultures of Venus-transduced cells depicted a clear expansion over a period of 3 weeks, either in the presence or absence of 4-OHT, ranging from 4.5-fold expansion in AML no.9 (AML no. 7 and no. 3 demonstrated a ~88-fold and ~28-fold expansion respectively, table 2, fig. 2A) to a ~860-fold expansion in AML no. 8 (table 2).

C/EBPα-ER-transduced cultures demonstrated a similar expansion curve in the absence of 4-OHT. Addition of 4-OHT, to activate the C/EBPα transgene in these cultures, immediately led to a strong reduction of expansion of all AML cultures (N=7, table 1), reflected by a reduction of cumulative cell counts and a sharp decrease in the percentage of Venus expressing cells in these cultures (fig. 2A and data not shown). Microscopic examination of the cultures verified these observations (fig. 2B). Sorting of Venus positive cells from expanding untreated control and C/EBPα-ER cultures and stimulating them with 4-OHT, rapidly induced a growth arrest in C/EBPα-ER transduced cultures only, indicating a cell-intrinsic effect due to C/EBPα activation, rather than differences between growth cultures (data not shown).

Since growth arrest of hematopoietic cells is frequently accompanied by differentiation, flow cytometric analysis on suspension cells was performed after 1 week of culture, to investigate the differentiation state of the leukemic CD34$^+$ cells. Analysis of myeloid markers specific for monocytic /macrophage (CD14) and granulocytic (CD15) differentiation demonstrated an increase in the percentage of cells positive for CD14 in C/EBPα-ER transduced cells as compared to control cells or to untransduced (Venus negative) cells within the same culture, ranging from 2% to 73% (fig. 2C and table 2). CD15 expression increased in 5 AMLs upon C/EBPα activation, ranging from 7% to 41%, whereas 2 AML showed a decrease of 2 and 5% of CD15 expression (table 2). Whereas almost all investigated AMLs showed changes in the expression of CD14 and CD15, in some of those CD36 changed also (table 2). In 3 AML cases (no. 8, no. 10, no. 11) CD11b was investigated as well and demonstrated markedly increased expression (table 2). In addition, a decrease

in the percentage of CD71 positive cells in C/EBPα-ER activated cultures was observed, compared to control Venus transduced cultures, (fig. 2C and table 2), although the degree of reduction ranged from 5% to 54% between AML samples (table 2). This is in agreement with the observed growth arrest in expanding cultures, since CD71 is a marker of proliferating cells.[429] RT-PCR studies demonstrated that C/EBPα-ER enhanced the expression of the C/EBPα target gene neutrophil elastase, which is induced upon granulocytic differentiation (fig. 2D).[430]

Morphological analysis of May-Grunwald-Giemsa stains confirmed the flow cytometry data regarding enhanced differentiation in some of these AMLs (fig. 2E). Control Venus-transduced cells of AML no. 3 (FAB M5) showed an immature monocytic morphology, which did not change upon treatment with 4-OHT. C/EBPα-ER-transduced cells however, showed the presence of more macrophage-like cells in untreated cultures, which was markedly increased upon activation of C/EBPα-ER wit 4-OHT. Together these data suggest that the absence of C/EBPα in AML cells contributes to the immature phenotype of these cells, which can be rescued partially by restoration of C/EBPα activation.



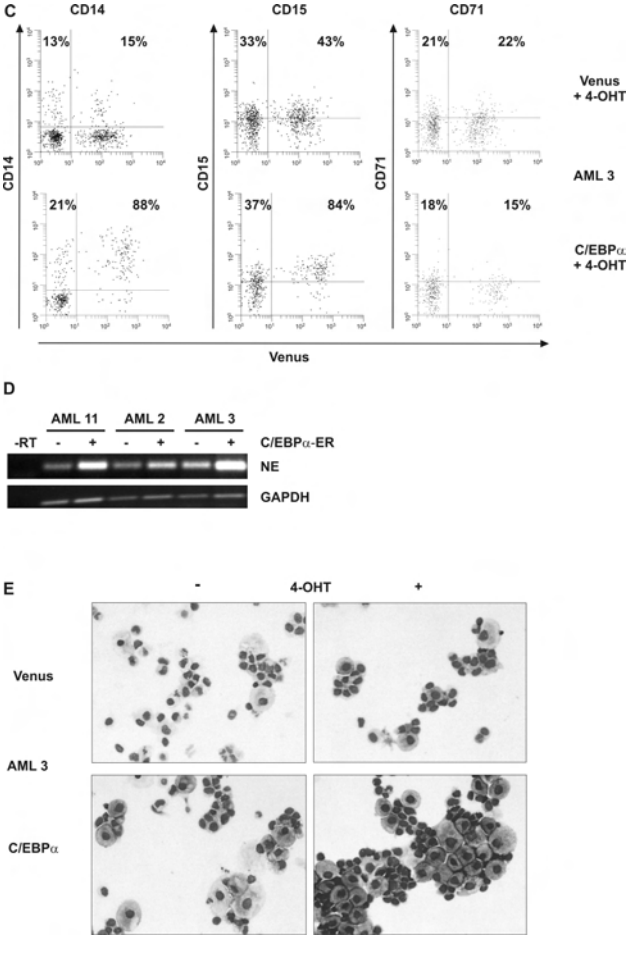


Figure 2 C/EBPα-ER impairs growth and expansion and induces differentiation of leukemic cells.
(A) Cumulative growth cultures of AML cells (N=7) on MS5 cells in the presence or absence of 500 nM 4-OHT. Cultures were demidepopulated weekly and fresh medium was added to the culture. (B) Representative phase contrast microscopy images of AML cocultures in the presence or absence of 500 nM 4-OHT on MS5 cells at weeks 3. Note the absence of phase bright suspension cells from C/EBPα-ER transduced culture after treatment with 500 nM 4-OHT. One representative example is shown. (N=7)
(C) Flow cytometric analysis of suspension cells from week 1 leukemic MS5 cocultures in the presence of 500 nM 4-OHT. One representative example is shown. Also refer to table 2. Indicated are the percentages of CD14, CD15 or CD71 positive cells within the Venus$^-$ or Venus$^+$ fractions. (N=7).
(D) RT-PCR analysis of Neutrophil Elastase (NE) expression in 3 AML samples after treatment with 500 nM 4-OHT in Venus-transduced cells (-) or in C/EBPα-ER-transduced cells (+). GAPDH expression is shown as loading control. -RT PCRs were performed as negative control. (E) Morphological analysis of suspension cells at week 1 of AML no. 9 was performed by MGG staining of cytospins.

Restoration of C/EBPα activation in primitive leukemic cells decreases the self-renewal capacity

In order to investigate the self renewal potential, we conducted serial replating experiments with the expanding AML cultures (N=6). Sorted CD34$^+$AML cells were transduced with C/EBPα-ER or empty control vectors and plated onto MS5 stroma. Every two weeks, the suspension cells were removed from the cultures and the adherent cell layer was washed two times and subsequently trypsinised. Leukemic cobblestone forming cells (L-CA) are present in this adherent fraction and contain self-renewal capacity as demonstrated by the capacity to generate new L-CAs upon each sequential replating onto new stromal cell layers (van Gosliga et al., submitted manuscript). 10% of the

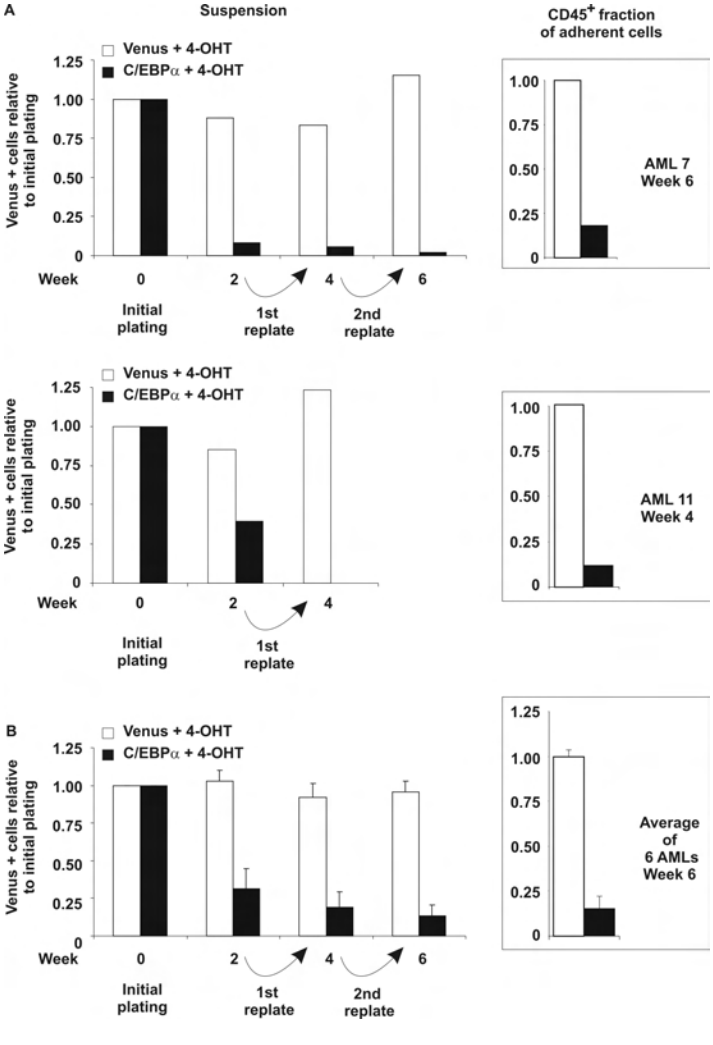
adherent AML cells were replated and after two subsequent weeks of culture the percentage of Venus-expressing cells was determined in the suspension fraction and within the $CD45^+$ fraction of the trypsinised adherent MS5 cell layer.

Two representative replating experiments (AML no.7 and AML no.11) as well as the average of 6 independent AML experiments are shown in fig. 3A and B. AML no.7 was transduced with Venus (Efficiency 72.3% at initial plating and set to 1) and C/EBPα-ER (Efficiency 46.7% at initial plating and set to 1). At week 2, the expansion of $Venus^+$ cells was comparable to expansion of non-transduced cells and thus the relative number of $Venus^+$ cells in suspension remained stable (fig. 3A). The adherent L-CAs that had formed were replated onto new MS5 stroma and these cells readily gave rise to 2^{nd} L-CAs (fig. 3C) that were capable of generating progeny in suspension (fig. 3A and B). Again, at week 4 no differences were observed in the expansion of $Venus^+$ cells versus untransduced cells in suspension, and the 2^{nd} L-CAs could be harvested to give rise to 3^{rd} L-CAs upon 2^{nd} replating (fig. 3A, B and C). At week 6, the relative number of $Venus^+$ cells was determined in both the suspension as well as adherent fraction, and in both populations $Venus^+$ cells behaved comparable to non-transduced cells. Similar results were obtained for AML no.11 (fig. 3A, second panels) and the average of replating experiments of 6 AMLs is also shown (fig. 3B).

In contrast to control Venus-transduced cells, the relative number of C/EBPα-ER-transduced cells in suspension dropped significantly as compared to untransduced controls (fig. 3A and B). This coincided with a reduction in the number of L-CAs that were formed in the initial plating (data not shown). Importantly, those remaining L-CAs were impaired in their replating capacity as no expanding 2^{nd} cultures could be established with these cells and strongly reduced numbers of C/EBPα-ER^+ cells were detected upon each replating, both in suspension as well as in the adherent population (fig. 3A and B). While 2^{nd} and even 3^{rd} L-CAs were readily observed in Venus control cultures, these L-CAs were absent from replated C/EBPα-ER cultures (fig. 3C). Collectively, these data indicate that restoration of C/EBPα activity in primitive AML $CD34^+$ cells decreases their self-renewal capacity.

In view of the declining self-renewal potential we studied expression of self-renewal genes such as BMI-1 and Meis1, which are highly expressed in $CD34^+$ primitive leukemic cells.[186;431] Tranduced cells were sorted after 1 week of MS5-coculture and subjected to RT-PCR studies. Of three investigated AML samples (AML no.11, no.2 and no.3), only AML no.3 demonstrated a decrease in expression of BMI-1 upon introduction of C/EBPα (fig. 3D). The same AML, also demonstrated lower Meis1 expression, while Meis1 expression was

unaltered in the other two AMLs investigated (fig. 3D). Furthermore, in view of the reduced proliferation capacity, the expression of the cyclin dependent kinase inhibitors (CDKi) p16, p18, p21 and p27 was investigated. Although no effects were seen on the expression of p18 and p27 in all three AMLs investigated (data not shown), the CDKi p16 showed an upregulation after C/EBPα-ER activation in AML no.3, while no effect was observed in the two other AMLs (fig. 3D) Expression of p21 was absent AML no. 11, while present in the other two AMLs. This expression was downregulated upon activation of C/EBPα (fig. 3D).



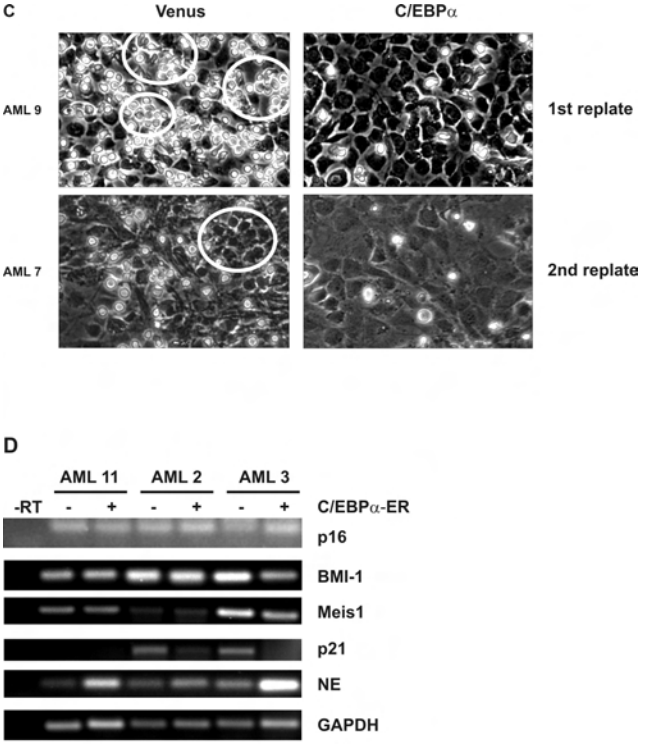


Figure 3 C/EBPα-ER impairs self-renewal of leukemic cells.
(A) Transduced AML cells were subjected to serial replating experiments in the presence of 500 nM 4-OHT (N=6) with cells from the adherent fraction. Every two weeks suspension cells were removed and analyzed by flow cytometry for Venus expression. 10% of the adherent fraction was replated onto fresh MS5 cultures. The Venus percentage within the human $CD45^+$ fraction was analyzed as well (Boxes on the right). Two representative experiments are shown (upper and middle panel). Venus percentages in suspension cultures at initial plating (Week 0) are set to 1 and subsequent weeks are shown relative to initial plating. The relative number of Venus + cells in the $CD45^+$ adherent fraction is shown relative to control Venus-transduced cells. (B). The average with SEM from 6 individual AMLs is shown. Venus percentages in suspension cultures at initial plating (Week 0) are set to 1 and subsequent weeks are shown relative to initial plating. The relative number of Venus + cells in the $CD45^+$ adherent fraction is shown relative to control Venus-transduced cells. (C) Representative phase contrast microscopy images of AML cocultures in the presence of 500 nM 4-OHT on MS5 cells after the first replate at week 4 (upper panels, AML no. 10), or after the second replate at week 6 (lower panels, AML no. 7). Cobblestone areas are encircled. (D) RT-PCR analysis of p16, BMI-1, Meis-1, p21 and Neutrophil Elastase (NE) expression in 3 AML samples after treatment for 1 week with 500 nM 4-OHT in Venus-transduced cells (V) or in C/EBPα-ER-transduced cells (C). GAPDH expression is shown as loading control. -RT PCRs were performed as negative control.

Discussion

Impairments in C/EBPα signaling such as reduced mRNA or protein expression, aberrant phosphorylation profiles, or the presence of (dominant negative) mutations are often observed in human myeloid leukemias.[81;254-257;260;261] We studied DNA-binding activities of C/EBPα throughout a panel of AML patients and showed that C/EBPα DNA-binding was reduced. On the basis of our rescue experiments in which C/EBPα was reintroduced into primary human $CD34^+$ AML blasts we conclude that restoration of C/EBPα expression in AML blasts is sufficient to inhibit both leukemic expansion as well as self-renewal of the leukemic cobblestone area (L-CA)-forming stem cell pool. In concert with the pronounced proliferation block, a partially restored differentiation program was observed by C/EBPα reintroduction.

Our data suggest that loss of C/EBPα function facilitates the self-renewal capacity of leukemic stem cells. In our C/EBPα-transduced cultures, leukemic cobblestone areas (L-CAs) were only rarely observed. Serial replating experiments established an impaired self-renewal potential as 2^{nd} and 3^{rd} cultures could mostly not be initiated. In contrast, the L-CAs that were generated from control-transduced cells contained self-renewal capacity as demonstrated in serial replating experiments. New L-CAs were generated upon sequential replating onto new bone marrow stromal cell layers and a robust leukemic expansion was obtained. Mouse knockout studies have already shown that C/EBPα deficiency enhances the self-renewal and repopulating capacity of HSCs. This results in hyperproliferation of hematopoietic progenitors and blocks the transition from the CMP to the GMP stage.[253;424] In normal human stem/progenitor cells, overexpression of C/EBPα resulted in exhaustion of the stem/progenitor cell pool, while myelopoiesis is induced at the expense of erythropoiesis[244;432] (and unpublished observations by ATJW, HS, EV and JJS). Together, these observations firmly establish C/EBPα as a critical regulator of stem/progenitor cell fate in both mice and humans.

Recently, C/EBPα was also reintroduced into primary Chronic Myeloid Leukemia (CML) cells at blast crisis, and although self-renewal of the stem cell compartment was not addressed upon C/EBPα reintroduction, myelopoiesis was restored upon IL-3 or G-CSF treatment.[433] These data coincide with our observations in our primary AML models in which myelopoiesis was also partially restored upon re-expression of C/EBPα. Besides these primary human leukemic models, a number of - mostly mouse - leukemic cell-line models have been established in which it was demonstrated that forced expression of C/EBPα induced cell cycle arrest, inhibited proliferation and induced

differentiation.[259;433] Previously, we demonstrated that expression of activated STAT5 in human CD34$^+$ stem/progenitor cells resulted in enhanced self-renewal and impaired myelopoiesis. This coincided with a strong downmodulation of C/EBPα expression.[37] Importantly, re-expression of C/EBPα in this model strongly alleviated STAT5A(1*6)-induced self-renewal, while myelopoiesis was restored.[244] Together, these data firmly establish the importance of disturbed C/EBPα signal transduction in a variety of hematological malignancies and pinpoint to the therapeutic potential of restoring C/EBPα activity in both acute and chronic myeloid leukemia.

As it is likely that disturbed C/EBPα signaling is only one of multiple factors involved in the pathogenesis of acute myeloid leukemia, it is not surprising that not all tested AMLs demonstrated a similar pattern. All investigated AMLs responded with a growth arrest and decreased expansion, while restoration of the differentiation program was much more variable, suggesting that cell-cycle arrest and differentiation are not affected in a similar fashion by the leukemic transformation. Experiments with various mutants of C/EBPα have indeed established that cell-cycle arrest is required, but not sufficient for granulocytic differentiation,[434] while the reverse experiment indicated that loss of cell-cycle control is sufficient to initiate AML-like transformation.[435] When (partial) differentiation was restored, in most cases both CD14 and CD15 were upregulated. This is consistent with findings that C/EBPα controls the CMP to GMP transition, but is not required beyond the GMP stage.[253] Therefore, it is not surprising that monocytic differentiation can be observed upon activation of C/EBPα. While C/EBPα deficiency had already been shown to block macrophage development,[424] only recently was C/EBPα shown to direct monocytic commitment, presumably through upregulation of PU.1 mRNA.[247;436] Although we did not detect an upregulation of PU.1 mRNA in that particular AML, preliminary observations in CB CD34$^+$ transduced cells with C/EBPα indicated elevated expression of PU.1 (data not shown).

The question by which mechanisms low C/EBPα levels enhance self-renewal of stem cells remains to be addressed. It was demonstrated that C/EBPα$^{-/-}$ mice express elevated levels of BMI-1 in Fetal Liver Lin$^-$/Sca-1$^+$/C-Kit$^+$ hematopoietic stem cells.[253] BMI-1 has an essential role in controlling the proliferative activity of both normal and leukemic stem cells.[186] Mouse HSCs engineered to express both HOXA9 and MEIS1 develop leukemia and require BMI-1 to transplant the disease into secondary recipients.[186] Indeed, 3 AMLs investigated demonstrated high expression of BMI-1, although only in one AML we observed that C/EBPα re-expression resulted in reduced BMI-1 and MEIS1 levels. This suggests that although C/EBPα leads to a partial restoration of differentiation with a reduction in growth and self-renewal, this does not

necessarily involve BMI-1 or MEIS1, indicating variability in the mechanisms involved. In that particular AML, downmodulation of BMI-1 coincided with an upregulation of the CDKi p16 and a downregulation of the CDKi p21, which has been described to be upregulated by a complex of Meis1 with PBX1 and HOXA10.[437] Although downregulation of p21 does not correlate with an enhanced cell-cycle arrest, the absence of p21 has been described to lead to a loss of self-renewal of HSCs,[29] which is consistent with our findings. Interestingly, p16 (Ink4a) is upregulated in the AML where BMI-1 is downmodulated by C/EBPα activation. Loss of Ink4a-Arf in HSCs has been proposed to lead to a loss of reconstitution potential upon serial transplantation[438] and high levels of p16 have been correlated to high levels of C/EBPα.[251] However, future experiments will have to provide evidence for such a link.

In conclusion, we demonstrate that re-introduction of C/EBPα in primary $CD34^+$ AML cells leads to a rapid growth arrest, which in some cases is paralleled by an enhanced differentiation. Furthermore we demonstrate that C/EBPα activation in the leukemic stem cells leads to a loss of self renewal capacity, thereby indicating the important function of loss of C/EBPα in the event of leukemogenesis.

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Summarizing discussion and future perspectives

Chapter 9

Summarizing discussion

Acute Myeloid Leukemia (AML) is a clonal hematopoietic malignancy, characterised by the accumulation of immature blasts in the bone marrow (BM). Approximately 40% of all leukemias are AMLs (In the Netherlands, the annual incidence is ~1/40.000 persons) and without treatment AML patients rarely survive more than 6 to 12 months. Treatment of AML patients usually consists of high intensity chemotherapy to induce complete remission (CR), followed by consolidation chemotherapy which might include an autologous or allogenic stem cell transplantation. However, the majority of the patients develop relapse of the AML, although the relapse rate strongly depends on the risk group. Patients belonging to the low-risk group have a relapse rate of ~25%, while patients of the high risk group have a relapse rate of ~80%.[20;439] These results indicate that cellular properties of the AML cells strongly dictate the patient's perspectives. In this thesis various aspects of AML were investigated: the development of AML and the difference between chemotherapy-resistant AML cells over normal stem/progenitor cells.

In **chapter two** we examined the involvement of Ras signal transduction in the activation of Nuclear Factor of Kappa B (NF-κB). First we demonstrated that NF-κB is activated in 73% of the AML patients. Subsequently, we provided evidence for an anti-apoptotic role of NF-κB, since AML samples with high NF-κB activity demonstrated a lower rate of spontaneous apoptosis, as well as a reduction in etoposide-induced apoptosis. This etoposide-induced apoptosis could be enhanced by inhibition with the NF-κB inhibitor SN-50. We demonstrated that constitutive Ras activation correlated with NF-κB activation, which could be inhibited by the Ras farnesyl-transferase inhibitor L744328 and the PI3K inhibitor Ly294002. Inhibition of MEK-Erk signalling with the inhibitor PD98059 did not affect NF-κB activity, indicating that downstream of Ras, PI3K signalling played a more important role than MEK-Erk signalling. In addition, PKB, the downstream effector of PI3K, was activated in AML blasts with constitutively activated NF-κB, suggestive for a Ras-PI3K-PKB signal transduction cascade upstream of active NF-κB. L744832 and Ly294002 inhibition indeed led to reduced phospho-PKB levels. The enhanced Ras activation was in part due to *Ras* mutations. The autocrine / paracrine secretion of growth factors might be an alternative pathway for Ras activation, but we did not detect activation of NF-κB via such a pathway. Finally, we demonstrated that constitutive NF-κB DNA-binding activity correlated with constitutive Flt3

phosphorylation. Although Flt3 signal transduction could be impaired with the inhibitor AG1296, this did not affect the NF-κB DNA-binding. This suggests that Flt3 signal transduction is sufficient to induce NF-κB activity, but it is not required and other signal transduction pathways also influence the activity of NF-κB.

These results appear to be in contrast to a recent report, where Flt3 activation was shown to activate IL-6 expression through NF-κB.[440] In that report, NF-κB activation was more dependent upon a MEK-Erk pathway than on PI3K-PKB. Although we detected no differences in NF-κB DNA binding upon MEK-Erk or Flt3 inhibition, Takahashi *et al*[440] reported similar findings, but they stated that the expression of NF-κB target genes is abrogated. This suggests that different pathways downstream of Flt3/Ras cooperate. PI3K-PKB is necessary for DNA binding of NF-κB, whereas MEK-Erk might be necessary for transactivation of NF-κB. Indeed, it has been demonstrated that overexpression of Ras can activate an NF-κB reporter, which can be abrogated by both MEK-Erk and PI3K inhibitors.[264;440] These and our findings are hence consistent with other reports stating that phosphorylation of different sites in p65 are involved in release, translocation, DNA-binding and transactivation of NF-κB.[441-443] Our data demonstrate that NF-κB DNA-binding is frequently activated by a Ras-PI3K-PKB pathway in AML cells and interferes with chemotherapy-induced apoptosis, but the precise signals that elicit Ras activation seem to be variable.

To investigate whether active NF-κB could play a role in the disturbance of steady state hematopoiesis of normal Hematopoietic Stem Cells (HSCs), in **chapter three** we introduced active mutants of NF-κB into cord blood-derived hematopoietic stem and progenitor cells. Since phosphorylation of p65, one of the major subunits of NF-κB in AML, plays an important role in the activation of this transcription factor, we designed and constructed various activating point mutants of both p65 and the Inhibitor of IκB Kinase 2 (IKK2), its upstream kinase. The activity of these mutants was investigated in various activation assays, such as reporter studies, DNA-binding experiments and quantative RT-PCR studies. This indicated that overexpression of wildtype p65 or the constitutive active mutant of IKK2 resulted in activation of the NF-κB pathway. Subsequent retroviral overexpression of these mutant proteins in cord blood-derived $CD34^+$ hematopoietic stem/progenitor cells was followed by various culture assays to assess whether proliferation, differentiation, self-renewal or apoptosis were affected. In long-term culture assays on bone marrow stromal

cells, we demonstrated that constitutive activation of the NF-κB pathway is not sufficient to impose a proliferative advantage on these stem/progenitor cells. Moreover, no differentiation block could be demonstrated. These results were confirmed in various colony assays, specific for common myeloid progenitors (CMP), megakaryocytic/erythroid progenitors (MkEP) and granulocyte macrophage progenitors (GMP) respectively. Finally, we addressed the question whether activation of NF-κB could enhance the number of primitive long term culture-initiating cells (LTC-IC), representative for HSCs. In these experiments we did not find evidence for a role of NF-κB activation in disturbing steady state hematopoiesis.

These experiments demonstrated that cells with activated NF-κB behaved similarly to normal control cells. This suggested that, although NF-κB activation may play an important part in resistance to chemotherapy-induced apoptosis (**Chapter two**), as a single hit, NF-κB activation is not sufficient to transform primary hematopoietic stem/progenitor cells. This is consistent with a report by Romano et al,[299] suggesting that NF-κB is not necessary for basal cell survival. The effect of NF-κB activation might be elicited when exposed to stress (chemotherapy) or, alternatively, might only be observed in combination with a second or third hit. Double transductions of constitutive active NF-κB with oncogenes/translocation such as for example AML-ETO, should be performed to gain more insight in such a hypothesis. It is important in such studies that second hits are chosen that do not activate NF-κB themselves, like for example Ras or Flt3. Although multiple studies have suggested that NF-κB plays an important role, most of these have used the strategy of inactivating NF-κB. Work in (cell-line) models that have been selected for NF-κB activation, and hence probably depend on it, not surprisingly lead to this conclusion, but are incapable of discriminating between additive or synergistic effects of NF-κB activation.

In primary monocytes, the cyclin-dependent kinase inhibitor p21 had been described to be a downstream mediator of NF-κB-induced inhibition of apoptosis. Since NF-κB is so frequently activated in AML, in **chapter four** we investigated whether p21 was involved in resistance to apoptosis of monocytic leukemia. We demonstrated that p21 was not expressed in unstimulated hematopoietic cells, but could be induced by growth factors, such as GM-CSF, IL-3 or PMA in an NF-κB-independent manner. Although expression of p21 could not be inhibited by U0126, Ly294002 or SB203580, inhibitors of the MEK-Erk, PI3K and p38 kinase pathways respectively, it could be inhibited by

BIM and Chelerythrin, inhibitors of PKC. When we investigated p21 expression in primary cells from monocytic FAB M4/M5 leukemias, we found that p21 was constitutively expressed in the majority of the M4/M5 leukemias, this in contrast to AML cells from M1/M2 leukemias. Furthermore, p21 appeared to be exclusively localised in the cytoplasm, which has been demonstrated to be necessary for its anti-apoptotic function. Indeed, we demonstrated that cytoplasmic p21 in M4/M5 leukemias was correlated to a decreased percentage of apoptotic cells. In order to unravel signal transduction pathways involved, we demonstrated that in some AMLs p21 expression could be inhibited by BIM and Chelerythrin, suggesting that activation of a PKC-dependent pathway is required. This is consistent with a report showing that PKC phosphorylates p21 and directs its nuclear exclusion.[444] Conditioned medium, which was used to culture cells from M4/M5 leukemias, was added to leukemic cell cultures from myeloid M1/M2 classifications to investigate whether autocrine production of growth factors was responsible for PKC-mediated p21 expression. These studies indicated that autocrine growth factor production might be involved in this activation. In addition, induction of p21 in a monocytic leukemia-derived cell-line inhibited apoptosis by inactivation of the MAPKinases JNK and p38. This suggested that cytoplasmic p21 might inhibit the upstream kinase ASK1. Immunoprecipitation assays from monocytic leukemia extracts indicated that p21 forms a complex with ASK1, which led us to propose that in monocytic leukemias part of their resistance to apoptosis is mediated via cytoplasmic p21. This then interferes with activation of p38 and JNK pathway-mediated apoptosis by inhibition of ASK1.

The effects of cytoplasmic localisation of p21 are not only observed at the level of apoptosis. Our own observations with a p21 variant that lacks the nuclear localisation signal (NLS) indicate that it no longer is capable of inducing growth arrest, which is consistent with other reports.[445] Thus the cytoplasmic localization of p21 in M4/M5 leukemias may also contribute to a growth advantage, due to the lack of a negative feedback system. P21 knock-out HSCs have a decreased self-renewal property,[29] which is correlated with an increased cell-cycle progression. Based on these findings it is of interest to investigate whether FAB M4/M5 monocytic leukemias have an altered self-renewal capacity or *in vitro* growth characteristics as compared to FAB M1/M2 myeloid leukemias. Expansion of AML cells on MS5 stroma indeed suggested that M4/M5 leukemias initially expand faster than M0-M2 leukemias, but after 10 weeks of long-term culture this difference disappeared (D. van Gosliga, J.J. Schuringa personal communication, manuscript submitted). It is however unclear whether this is due to p21 localisation and hence effects on growth or self renewal, or an inherent property of monocytic AMLs. Furthermore, it can

not be excluded that p21 only affects apoptosis and cell-cycle and not self-renewal, since the self-renewal phenotype of p21-deficient HSCs appears to be mouse strain-dependent.[185] It would be interesting to study the cellular properties of CB-derived $CD34^+$ cells transduced with a p21 construct lacking the NLS. Similar, it might be relevant to study the effects of PKC inhibitors on the growth characteristics of AML cells. It is known that the PKC-inhibitor UCN-01 with various other inhibitors can enhance the apoptotic potential of monocytic U937 cells, but UCN-01 alone has marginal effects regarding apoptosis.[446-448] This would thus make it possible to study the effects of p21 on cell-cycle and self-renewal in the absence of apoptosis in both normal cells as well as in monocytic leukemias.

The above described DAXX-ASK1 pathway has also been shown to be modulated by chaperone proteins such as HSP27. Since HSP27 had been demonstrated to be upregulated in AML blasts, in **chapter five** we investigated the role of Heat Shock Protein 27 (HSP27) in compensating apoptosis in AML cells via the ASK1-p38-JNK pathway. First we demonstrated that downregulation of HSP27 by means of RNA interference (RNAi), increased etoposide-mediated apoptosis in leukemic-derived TF-1 cells. To explore whether HSP27 exerted its effects downstream in the apoptotic pathways or upstream, co-immunoprecipitation studies were performed to study its interaction with pro-caspase 3, cytochrome c and DAXX. We failed to detect HSP27 in complex with either pro-caspase 3 or cytochrome c, which suggested that HSP27 exerts its effects higher up in the apoptotic pathway. Complex formation with DAXX confirmed this suggestion and this interaction depended upon the presence of ASK1. Since cytochrome c release into the cytoplasm was enhanced after HSP27 RNAi, we examined the effects on phosphorylation of the upstream kinases involved in mitochondrial integrity, p38 and JNK. These are downstream of DAXX-ASK1 signalling. Western blot analysis revealed that upon downregulation of HSP27, activation of p38 and JNK (as measured through phosphorylation of the downstream target c-Jun) was enhanced upon treatment with etoposide. Together, we concluded that HSP27 is able to inhibit DAXX-ASK1-p38/JNK-mediated apoptosis.

The results described in **chapters four** and **five** demonstrate that p21 and HSP27 can associate with the same apoptosis-inducing protein complex, DAXX and ASK1. However, p21 and HSP27 were not simultaneously detected in the same complex. We examined whether HSP27 expression was correlated with decreased levels of apoptosis in primary AML cells, as the cell-line model predicted, but no correlation between HSP27 expression and apoptosis could be demonstrated. Interestingly, HSP27 expression correlated with p21

expression, both of which were mainly expressed in M4/M5 leukemias and absent in M1/M2 leukemias. The strong anti-apoptotic effect of cytoplasmic p21 might compensate for the absence of HSP27. This would seriously hamper attempts to correlate HSP27 expression to decreased levels of apoptosis. To test this, we created a TF-1 cell-line, which constitutively expressed a cytoplasmic form of p21, and performed HSP27 RNAi in these cells. Subsequent western blot analysis demonstrated that the enhanced activation of p38 after HSP27 RNAi could be impaired when p21 was expressed in the cytoplasm, providing evidence for our hypothesis.

Interestingly, HSP27 demonstrated only a cytoprotective effect after treatment with etoposide, but not with CD95/Fas. Although CD95/Fas treatment did result in pro-caspase 3 cleavage, arguing for a Fas-FADD apoptotic route independent of p38 and JNK, this did not result in apoptosis. Examination of HSP27 levels revealed that upon CD95/Fas treatment HSP27 levels were drastically increased. This is suggestive for an anti-apoptotic effect of HSP27 in the CD95/Fas pathway as well, in a way that CD95/Fas treatment induces HSP27 and thereby impairs its own apoptotic induction. The upregulation of HSP27 however was so massive, that attempts to downregulate HSP27 failed in this setting and we therefore could not further explore this hypothesis.

Leukemic cells have different properties that give cells a growth advantage over their normal counterparts. Besides defects in apoptotic pathways, the cells might be less susceptible for the effects of negative regulators. Therefore in **chapter six** we examined the mechanism through which N-Ras^{L61} transformed cells escaped negative growth regulation by TGF-β. Two Ras-transformed cell lines, HL-60 and TF-1, were compared to a cell line without the Ras mutation, M1 cells. These Ras cell lines were unresponsive to TGFβ with regard to a cell cycle arrest, whereas TGF-β-induced transcription was normal. Interestingly, p27 was absent from Ras-transformed cells, which suggested that p27 was the central protein through which TGF-β-mediated cell cycle responses could be modulated. Using the L744632 and U0126 inhibitors, we demonstrated that a TGF-β response could be restored, which coincided with restoration of p27 expression. Overexpression of p27 indeed was sufficient to restore cell-cycle arrest upon TGF-β treatment. Further investigations with transcription, translation and degradation inhibitors, established that Ras-MEK-Erk signal transduction was involved in degradation of the p27 protein, rather then suppression of transcription or translation. A well known protein involved in degradation of p27, SKP2, indeed proved to be upregulated in Ras-transformed cells, and this could be inhibited by inhibiting the Ras pathway

using the inhibitor L744832. Moreover, overexpression of N-Ras^{L61} in M1 cells resulted in an enhanced expression of SKP2. Further involvement of SKP2 in p27 degradation downstream of Ras signal transduction was demonstrated by overexpression of various p27 phosphorylation mutants. Interestingly, only mutation of the phospho-site known to be involved in SKP2-mediated degradation, left p27 resistant to degradation. This in contrast to observations that multiple sites were phosphorylated. U0126 treatment did not alter the phosphorylation status of p27, which was in line with our observations of an indirect effect of Ras-MEK-Erk signalling. Subsequent immunoprecipitation studies established complex formation between SKP2 and p27, and RNAi against SKP2 restored p27 levels in these cells. These data demonstrated another mechanism by which Ras signal transduction could render cells unresponsive towards the negative regulatory effects of TGF-β and contribute towards the advantage of leukemic cells over normal cells.

In four AML patients with N-Ras^{L61} mutations, we indeed observed absence of p27 or mislocalisation to the cytoplasm. In other studies mislocalisation of p27 in AML patients was attributed to PKB phosphorylation,[155] which is consistent with Ras activation as observed in **chapter two**. However, in our cell line models we could not correlate p27 phosphorylation with PKB activation. In those AML patients with PKB activation, the poor disease free and overall survival was not correlated to the level of p27 protein, but to a high cytoplasmic to nuclear ratio. Although these results seem different from our model it may actually result in a similar phenotype, since p27 degradation in the nuclear compartment will yield a high cytoplasmic to nuclear ratio. Indeed, Min *et al* did detect a heterogeneous pattern of p27 localisation, arguing that different models can be present in AML. Furthermore, in conjunction with high cytoplasmic to nuclear ratios of p27 they also demonstrated that SKP2 was highly expressed in the majority of the AML patients and correlated to both poor disease free survival and overall survival.[401]

The above described degradation of p27 was attributed to the activation of the Ras-MEK-Erk pathway, but in **chapter two** we demonstrated that Ras activation is not necessarily due to *Ras* mutations. Mutations in the Flt3 receptor have been demonstrated to lead to Ras-MEK-Erk activation as well. These mutations are very common in AML (~30%) and frequently also activate PI3K and STAT5 signal transduction pathways. To examine the effects of STAT5 on the cellular properties in normal primary cells, in **chapter seven** we lentivirally introduced a RNAi hairpin against STAT5 in CD34^{+} CB-derived

stem/progenitor cells. This enabled us to downregulate STAT5 protein levels by up to 85%. In long-term cultures with stromal bone marrow cells, we established that downregulation of STAT5 severely reduced expansion of CB $CD34^+$ cells, without altering the differentiation capacity. This in contrast to overexpression of an activated form of STAT5, which skewed differentiation of progenitors towards an erythroid fate.[37] Interestingly and paradoxically, the observed reduction in expansion was not due to an increased apoptosis or an increased cell-cycle arrest in bulk cultures. Limiting dilution analysis of both progenitor numbers as well as the more primitive LTC-IC, indicated that STAT5 knockdown resulted in a 5-fold reduction in progenitor and LTC-IC frequency. To further assess how the primitive cells were affected, $CD34^+CD38^-$ cells were transduced and followed individually in culture for 100 hours by microscopic examination. The results indicated that transduction of STAT5 RNAi reduced the number of cell divisions with a subsequent reduction in offspring production, compared to control cells. In line with the limiting dilution progenitor assays, this eventually resulted in a lower output of colony numbers. Finally, STAT5 downregulation was studied in primary AML cells. $CD34^+$-sorted AML cells were transduced with the STAT5 RNAi lentiviral vectors, long-term cultures were initiated, and we observed a reduced expansion of these cells similar to what was observed in CB-derived $CD34^+$ cells. Furthermore, FACS analysis demonstrated no effect on the differentiation pattern of these AML cells.

Our observations regarding differentiation correspond to the relatively mild phenotype of STAT5A/$B^{-/-}$ mice. These mice displayed normal levels of all blood cell types in the peripheral blood.[239] In contrast, a significant reduction in progenitor and LTC-IC frequencies was observed by downmodulation of STAT5 expression in the HSC and progenitor subpopulations. The results are compatible with the observation published by Scherr *et al,* who have reported similar reductions in $CD34^+$ progenitors.[411] Furthermore, their and our observed reduction in colony numbers after STAT5 RNAi corresponded with the reductions seen in CFU-mix colonies and in CFU-GM colonies in STAT5$AB^{-/-}$ mice.[239] These data demonstrate that very primitive hematopoietic cells are dependent upon STAT5 for normal growth. It suggests that leukemic stem cells benefit from constitutive STAT5 activation, which is frequently observed in AML. Although overexpression of a constitutively activated STAT5 can skew differentiation towards an erythroid fate, downregulation does not affect this differentiation, arguing that either STAT5 is sufficient, but not necessary, or that redundancy exists among STAT family members. The possibility that RNAi is not effective enough in downregulating STAT5 is highly unlikely, given the similar phenotypes of the STAT5$AB^{-/-}$ mice. Overexpression of STAT5 also

enhances the self-renewal potential of HSCs, which is consistent with the decreased repopulation potential of STAT5AB$^{-/-}$ HSCs.[240;241] Future experiments will be focused on self-renewal properties by replating assays with RNAi-transduced leukemic cells.

It was recently demonstrated that activation of STAT5 results in downregulation of CCAAT enhancer binding protein alpha (C/EBPα) and re-introduction of C/EBPα restored progenitor cell differentiation.[244] Different studies have recently shown that C/EBPα is frequently mutated or otherwise inactivated in AML cells. In **chapter eight** we therefore examined the effects of re-introduction of C/EBPα in primary AML stem cells. First we demonstrated that C/EBPα DNA binding was severely diminished in CD34^{+} sorted primary AML cells, which underscored the reduced C/EBPα expression observed in various AMLs. Long-term culture on stromal bone marrow cells after lentiviral introduction of an inducible form of C/EBPα, demonstrated an immediate growth arrest upon induction. This growth arrest was accompanied by a partial restoration of differentiation, as measured by FACS analysis for markers demonstrating monocytic and granulocytic differentiation (CD11b, CD14 and CD15). In one AML, MGG stains also indicated the presence of more fully differentiated macrophages, confirming the observed FACS profile. Interestingly, almost no leukemic cobblestone area forming cells (L-CA), a measure for stem cell activity, were observed in C/EBPα induced cultures, whereas control cultures did. This suggested that the self-renewal property of LSCs might be affected, since it was already known that C/EBPα knockout HSCs show an enhanced self-renewal.[253] To test this, replating experiments were performed by sequentially passaging adherent leukemic cells onto fresh bone marrow stroma and subsequently the ability to repopulate a new leukemic culture was measured. Strikingly, whereas control cultures were able to start a new culture for up to 3 times at least, with the appearance of new L-CAs, C/EBPα induced cultures were incapable of doing so. Subsequently, self-renewal and cell-cycle genes were studied in response to C/EBPα restoration. In one AML, we demonstrated that C/EBPα activation correlated with the reduced expression of self-renewal genes BMI-1, Meis1 and the CDKi p21, whereas upregulation of the CDKi p16 was observed as well. This would fit with data from C/EBPα$^{-/-}$ HSCs. However, two other AMLs studied did not indicate such an effect of C/EBPα restoration on these genes, indicating that more experiments need to be performed to establish a link between C/EBPα restoration and the downstream events involved in decreasing the self-renewal capacity.

Interestingly, the AML that showed a reduction in BMI-1 and Meis1 expression upon introduction of C/EBPα also had the highest increase in differentiation markers, suggesting that differentiation and self-renewal are directly related to each other. Future experiments using more AML samples are required to generalize our findings, but it is interesting to note that there is not only a distinction between cell-cycle arrest (observed for all AMLs) and differentiation (observed in some AMLs), but also with self-renewal (decreased replating was observed for all AMLs) and differentiation. The fact that cell-cycle arrest is observed in all AMLs along with a decrease in self-renewal capacity, demonstrated that C/EBPα was functionally introduced into these cells. However, this is in sharp contrast to observations that self-renewal is impaired if cell-cycle progression is stimulated, as has been postulated for the $p21^{-/-}$ HSCs.[29] Experiments designed to modulate the expression level of C/EBPα would give more insight in the various functions of C/EBPα. Future studies should also include the analysis of a larger cohort of AML samples in which C/EBPα is re-introduced.

Models for signal transduction-targeted inhibitors

During the last decades the understanding of the genetic background, activated signal transduction pathways and signal transduction intermediates involved in the etiology of AML has expanded exponentially. Despite this enormous increase in the amount of knowledge and the subsequent progress in AML treatment, standard induction therapy still fails to induce remission in up to 20-30% of the AML patients. Furthermore, relapse is common in approximately 60% of these patients in remission.[449] Of adult AML patients up to 60 years of age, only 30% demonstrate long-term disease-free survival and this prognosis is much worse for older patients.[439] In the upcoming years, with an increasing mean age of our population, the number of AML patients is likely to rise and, without treatment improvements, AML-related morbidity and mortality as well.

Due to the low treatment success rate and the increased amount of knowledge regarding AML, in recent years a plea for more signal transduction-targeted therapies can be heard. The drugs that are currently entering (clinical) trials can be grossly divided into two classes: Inhibitors that target receptor tyrosine kinase signalling and inhibitors that interfere with genetic remodelling. In figure 1, these inhibitors are depicted along with some of their proposed actions.

Flt3 inhibitors, such as PKC-412, CEP-701, ML-518 and SU5416[439] are expected to enhance apoptosis and lead to cell cycle-arrest, since the downstream Ras pathway is inactivated, which will lead to a subsequent inactivation of NF-κB and an upregulation of p27. As STAT5 is implicated to be downstream of Flt3 in inducing early cobblestone formation,[450] effects are also expected on self-renewal and differentiation. Indeed, Zheng *et al* were able to demonstrate that inhibition of Flt3-ITDs using CEP-701, resulted in partially restored myelopoiesis, due to restoration of C/EBPα.[259] Recently, both STAT5 as well as MEK-Erk signalling have been shown to downregulate C/EBPα and may thus be responsible for these observed effects.[244;260]

Inhibitors that act on the Ras pathway, the so-called farnesyl transferase inhibitors, including R115777 (Tipifarnib or Zarnestra), L-778,123, BMS-214,662, CP-609,754 and SCH66336, have entered phase I and II trials[449] and are expected to act in a similar fashion. In contrast to both Flt3 and farnesyltransferase inhibitors that act upstream to inhibit these pathways, the proteasome inhibitor Bortezomib (Velcade), also interferes with growth and survival of AML cells through the upregulation of both p27 and the inhibitor of NF-κB protein IκB. Recently, a report suggested that prolonged exposure to Flt3 inhibitors leads to resistance, due to the activation of parallel pathways and bypassing the necessity of Flt3 for activation of downstream signal transduction components.[451] This suggests that inhibiting numerous downstream targets would be preferred over more upstream targets in these signal transduction pathways.

Two of the Flt3 inhibitors mentioned previously, PKC-412 and CEP-701, have also been used as PKC inhibitors,[439] which might be used to inhibit p21 expression in monocytic leukemias. The compound 7-hydroxystaurosporine (UCN-01), which is currently involved in various clinical I and II trials, has also been suggested to serve as a PKC inhibitor and indeed, its use has demonstrated to enhance JNK and p38 activation.[446-448;452;453] UCN-01 has furthermore been assigned a role in reducing PKB activation, which contributes to the induction of apoptosis along with activation of p38 and JNK.[447;448;452] However, the use of UCN-01 enhances Raf1-MEK-Erk signalling, but combinations with a farnesyltransferase inhibitor or the clinical HSP90 inhibitor 17-AAG, have been shown to reverse this effect and potentiate mitochondrial induced apoptosis and inhibition of NF-κB.[447;454] Although no effects on HSP27 have been described for both UCN-01 or 17-AAG, potentiating p38 and JNK would bypass HSP27-DAXX-ASK1 and lead to apoptosis.

The second group of inhibitors interfere with proteins that modulate the accessibility of the DNA, the histone deacetylases (HDACs) and DNA methyl

transferases (DNMTs). HDAC inhibitors (Valproic acid, SAHA, butyrate, depsipeptide and MS-275) and inhibitors of DNMTs, such as azacitidine and decitabine, are currently undergoing clinical trials.[455] Deacetylase activity has been demonstrated to be required for STAT5-dependent transcription.[456;457] Furthermore, translocation products in AML also use chromatin remodelling as a means of gene repression and in the majority of those C/EBPα is downregulated as well.[81;261;425;426] If these events are somehow linked, treatment with HDAC and DNMT inhibitors can restore C/EBPα expression and control constitutive activation of STAT5 in AML, with subsequent effects on self-renewal, differentiation and cell cycle. However, some caution should be taken using such inhibitors as well, since upregulation of p21 has been demonstrated to occur.[458] In monocytic leukemias this might have adverse effects, since it interferes with apoptosis, instead of potentiating it.

In analogy to the multiplicity of potential molecular targets in AML, many inhibitors have been designed and are currently under evaluation for their efficacy. Regarding the heterogeneity of the disease, it is likely that future therapy will involve a combination of various inhibitors, depending upon patient-specific parameters.

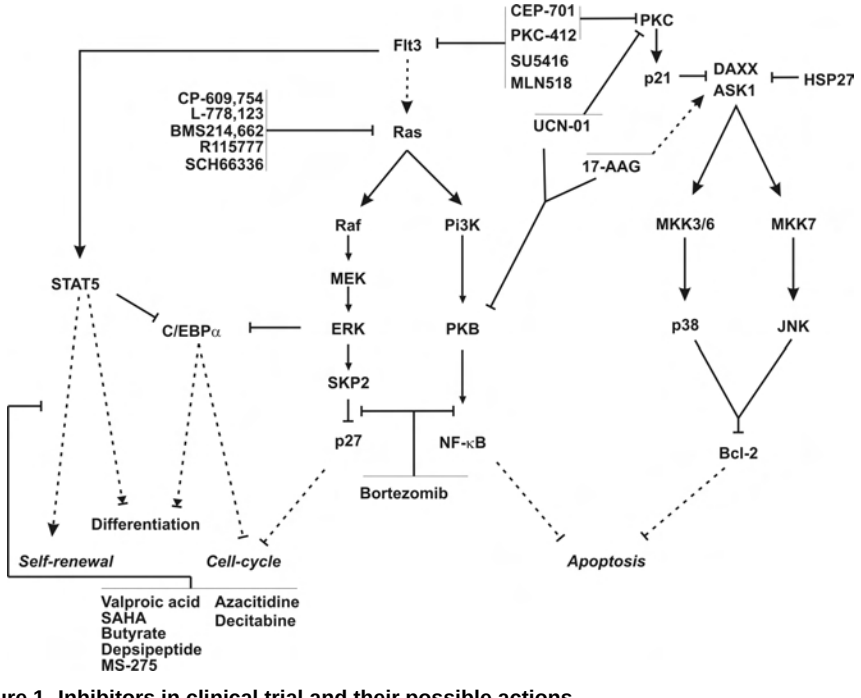

Figure 1 Inhibitors in clinical trial and their possible actions.
→indicates positive action, ⊥ indicates inhibitory action

Future perspectives

Based on genomic alterations in the process of leukemic transformation, a number of signal transduction pathways are affected that regulate apoptosis, proliferation, differentiation and self-renewal of AML cells. The current view of leukemic transformation involves mutations affecting differentiation and self-renewal as first hit (group 1 mutations), combined with second hit mutations that affect survival and proliferation (group 2 mutations). This model is also known as the multiple hit model. In some cases (chapter 8) it might appear that interfering with one single hit is sufficient to restore some elements of hematopoiesis. However, not all AMLs behaved in the similar way, indicating that multiple “hits” make use of different, yet unknown pathways.
The usage of PCR-techniques has provided additional information regarding clonal abnormalities, such as mutations of Flt3, c-Kit, Nucleophosmin (NPM), Ras and C/EBPα to the current cytogenetics, based on metaphase banding and FISH techniques, which only detect relatively gross abnormalities, including chromosomal translocations and deletions. However, heterogeneity is still common amongst groups of AML patients, even within groups with a defined genetic make-up, such as t(8;21)(q22;q22) or inv(16)(p12;q22).[459] In order to improve our current knowledge of AML, various approaches should be taken to further subdivide and increase the homogeneity of these subgroups in order to unravel the underlying mechanisms.

Gene expression profiling and PCR
A powerful tool in characterisation of large cohorts of AML patients is gene expression profiling. Valk *et al.* and Bullinger *et al.* identified seven novel subgroups of AMLs based on gene expression profiles.[460;461] The clustering of patients with known chromosomal aberrations, such as t(8;21) (AML-ETO), t(15;17) (PML-RARα) and inv(16) (CBFβ-MYH11), demonstrate the validity of this approach, but is hardly surprising, given the effects on gene expression of most of these translocations. However, the subgroups of patients with t(8;21), inv(16), 11q23 abnormalities as well as the AML subgroups with C/EBPα mutations, EVI1 expression and the AML subgroup with a normal karyotype were further subdivided in two or more classes. The explanation of this segregation within these groups lies probably in the fact that additional genetic defects add to a distinct gene expression profile. The challenge is therefore to characterise these additional hits and further subdivide into groups with a more homogenous character.

Based on this, both groups designed a set of predictor genes, with which they could predict clinical outcome in an unsupervised clustering analysis. Although both groups have some overlapping and some diverging results, it is interesting to note that a few genes are present in both of their predictor gene sets and may serve as novel targets for future therapy. Amongst these are homeobox genes of the HOX family as well as the homeobox gene PBX3. Whereas the role of HOX family members in self-renewal and leukemia becomes more and more understood, the precise role of PBX3 has yet to be discovered. Especially the PBX3C isoform is an interesting target since its expression is favoured in leukemic cells and may contribute to the process of leukemogenisis.[462] To assess this, overexpression of this isoform in cord blood stem and progenitor cells may reveal its involvement in the enhanced self-renewal capacity of leukemic progenitors.

Furthermore, members of the CTNNA1 and CTNNAL1 are amongst the set of predictor genes. These genes encode for catenin family members α-catenin and the α-catenin related protein α-catulin. Both proteins inhibit or attenuate the synergy between Ras and β-catenin and their expression may therefore play an important role in regulating catenin signalling.[463] Since the downstream mediator TCF4 was on one of the prediction sets and recently γ-catenin was implicated in leukemia,[464;465] it would be interesting to investigate the role of these regulators of catenin signalling in AML.

A third set of genes within these predictor sets with possible implications for our understanding of the leukemic clone are ANGPT1 and ANGPTL4. The angiopoietins and angiopoietin-like proteins were recently shown to affect hematopoietic stem cell expansion,[55;466] and it may therefore be rewarding to downregulate these proteins in the leukemic stem and progenitor cell compartment.

Although these findings demonstrate that gene expression profiling is a valuable prognostic tool and may indicate several relevant gene-products for drug targeting, knowledge about small (point) mutations can also be relevant. Although Valk *et al*. showed that gene expression from patients with C/EBPα mutations clustered together, they could not show similar results for patients with Ras or Flt3 mutations.[460] This either implicates that additional mutations add to the gene expression profile; or, the effects of high expression of wt genes resembles the downstream effects of the mutated gene. This latter possibility has been suggested for high wt Flt3 expression in relation to Flt3-ITD and prognosis.[461] In cases where Flt3 mutations associated with NPM1 mutations, the favourable outcome, correlated to NPM1 mutations, was lost.[96] Whether this is also true for high Flt3 expression in combination with NPM

mutations, needs further investigation. Since more than 50% of the patients with a normal karyotype have NPM1 mutations and approximately 30% have Flt3 mutations, combining gene expression profiling with mutation analysis may have prognostic value for the patients in this group.

Protein levels and activation

In defining risk-groups of AML patients, it may also be useful to know the activation status of the proteins involved. Gene expression does not always correlate with protein expression or activation, due to post transcriptional and post translational modifications. P27 for example, was expressed at the mRNA level in N-Ras mutated cell-lines, but was absent at the protein level due to enhanced degradation. We also observed that the expression of C/EBPα mRNA in various AMLs did not always correlate with the level of C/EBPα protein on DNA binding. Furthermore, some of the C/EBPα transduced AMLs did not differentiate, although all responded with a growth arrest. This suggests that the protein level or its activation is of utmost importance for the cell biological outcome.

Such dose-response outcome is supported by studies in mice with various expression levels of the transcription factor PU.1. Downregulation of PU.1 by 80% induces a pre-malignant state with an expansion of myeloid progenitors, this in contrast to knock-out PU.1$^{-/-}$ or heterozygous PU.1$^{+/-}$ mice.[467] This may also be important for human hematopoiesis and leukemia, since PU.1 is necessary in both MPP to CMP differentiation, as well as in lineage commitment via the CLP stage. Although mutations in the PU.1 gene (*Sfpi1*), affecting DNA binding or transactivation are found in only 7% of all AML patients,[468] multiple cytogenetic abnormalities have shown to result in PU.1 downregulation. Reintroduction of PU.1 into leukemic stem cells should be performed to confirm its involvement in the leukemic process and graded re-introduction might shed some light upon its regulation of cell-cycle vs. differentiation and self-renewal.

The above described gene expression profiling has implicated the homeobox family of HOX genes in the leukemic process[460;461] as well as in a recent model of MLL-AF9-induced leukemia.[106] Indeed, HOX expression enables the identification of a subset of AML patients with intermediate cytogenetics,[469] but the precise manner in which the HOX genes contribute is still unclear. Transient overexpression of HOXB4 in CB CD34^{+} cells has revealed that myeloid differentiation is promoted, whereas proliferation is not impaired.[470] On the other hand, continuous expression of various HOXB4 (protein) levels has demonstrated that high levels actually impair differentiation

and showed an enhanced proliferation.[471] This apparent dichotomy needs further clarification, since a better understanding may lead drug development towards lowering HOXB4 levels.

However, care should be taken when it comes to lowering proteins levels of highly expressed genes. The H-Ras protein for example, reduces proliferation of human hematopoietic progenitors and enhances monocytic differentiation when highly expressed. But upon farnesyl transferase treatment of these H-RAS expressing progenitors, not only their proliferation was increased, but their self-renewal as well.[472] Similar data has been observed in mice expressing PML-RARα, for which it was shown that the development of APL in mice could be enhanced from an incidence of 20% to 90%, when its expression was lowered.[473;474] Although these two models seem counterintuitive in their behaviour, e.g. lower activation, but enhanced leukemic potential, in combination with the other examples they nicely illustrate the point that activation levels itself hold important information regarding the outcome.

Data using 4-OHT-inducible STAT5-ER proteins suggest that the cell biological phenotype indeed highly depends on the level of STAT5 activity (A.T.J. Wierenga and J.J. Schuringa, personal communication) and observations that constitutive STAT5 activation in AML is lower than cytokine-induced STAT5 activation, further underscore the necessity of future investigations into modulating expression and activation levels of the proteins mentioned above. Obtaining knowledge regarding the proteomic make up of AML patient cohorts, should add to our current understanding of the heterogeneity of the disease.

Studying the leukemic stem cell compartment

Part of the heterogeneity in AML in the response to therapy may be due to the fact that the leukemic stem cell that gives rise to the observed bulk of blast cells is very rare. Although the observed bulk of blasts cells share some degree of similarity with the leukemic stem cell, e.g. mutations and translocations, high relapse rates indicate a failure to eradicate this cell and suggest that it is different then the observed blast cells. Since many studies of AML cells have involved blast cells, it may not always be possible to extrapolate the information from these studies towards the leukemic stem cell. This is in part due to our lack of knowledge regarding the leukemic stem cell and therefore it is of crucial importance to gain more insight into the nature of this cell. Bonnet *et al.* have greatly enhanced our view of the phenotype of this leukemic stem cell, in showing that this cell resides in the pool of $CD34^+$ $CD38^-$ cells and is capable of engraftment in immune deficient mice.[32] The phenotype of these NOD/SCID

engrafting cells, has since been extended to $CD34^+$ $CD38^-$ $CD33^+$ $CD123^+$ $CD71^-$ $CD90^-$ c-Kit^- HLA-DR^-.[102-104;475;476] Our leukemic long-term cultures have been performed with $CD34^+$ cells, in which the frequency of stem cells is already enhanced, but further fractionation would be desirable, in order to gain more insight in the events that drive the leukemic process.

The long term stromal co-culture system, as used in some of our experiments, is an easy accessible system to screen various fractions of leukemic cells for their clonogenic “stem cell” capacity, which subsequently must be evaluated by engraftment studies in NOD/SCID mice. However, approximately 50% of all AMLs do not engraft NOD/SCID mice, which has been correlated to the karyotype of the AML.[422] This might be due to the possible stem cell origin of those particular AMLs that do engraft and the possible progenitor origin of those that do not. Alternatively, it may be due to the transforming event, e.g. the resulting translocation product or mutation. It is worth while to investigate these possibilities by modulating translocation products, mutated proteins and other potential targets obtained from the above described gene expression and proteomic profiles. Transduction of AML stem cells with (inducible) RNAi vectors and investigating the differentiation and replating capacity *in vitro* and the engraftment of NOD/SCID *in vivo* should shed more light on the nature of the leukemic stem cell.

In conclusion, gene expression profiling as well as proteome expression profiling should provide us with potential targets for future studies. Interfering with these potential targets, preferably in leukemic (stem) cells, will provide us with a better understanding of their role in the various AML subtypes. This knowledge can subsequently be used to develop patient-tailored therapies as well as new clinical inhibitors, resulting in a better treatment and curability in the future.

Nederlandse samenvatting

Chapter 10

Hematopoiese

Hematopoiese is een hiërarchisch proces van bloedcelontwikkeling dat start bij de hematopoietische stamcel (HSC). Deze HSC is de moedercel, van waaruit alle dochterbloedcellen voortkomen. De HSC is zeer zeldzaam, wordt voornamelijk gevonden in het beenmerg en heeft eigenschappen waardoor het kan kiezen tussen zichzelf vernieuwen (een proces dat self-renewal heet), of veranderen (differentiëren) naar een van de vele dochtercellen die ons bloed rijk is. Self-renewal zorgt ervoor dat na deling minstens één HSC behouden blijft. Dit wil zeggen dat tenminste één van de ontstane dochtercellen de capaciteit behoudt om elke gewenste dochtercel te worden. Dit in tegenstelling tot het proces van differentiatie, waarbij de dochtercel dit vermogen verloren heeft en zich specialiseert in een bepaalde richting.

Het proces van hematopoiese voorziet het lichaam van miljarden bloedcellen per dag en moet flexibel genoeg zijn om zich aan te kunnen passen om de juiste cel op het juiste tijdstip te produceren. Hematopoiese is daarom een strak geregisseerd proces, waarbij extracellulaire mechanismen, zoals beïnvloeding door groeifactoren en cel-cel interacties, samen met intracellulaire mechanismen een belangrijke rol spelen. Veranderingen in het doorgeven van de daarbij behorende signalen (signaaltransductie) en de daaropvolgende veranderingen in de activiteit van genetische programma's zullen uiteindelijk leiden tot een zeer goed afgestelde balans tussen de processen die bij bloedcelontwikkeling een belangrijke rol spelen.

Acute myeloide leukemie

Acute myeloide leukemie (AML) is een klonale hematopoietische afwijking in de bloedcel-ontwikkeling, die gepaard gaat met ophoping van onrijpe blasten (vroege cellen) in het beenmerg. Deze blasten zijn verstoord in hun vermogen om te differentiëren tot verschillende uitgerijpte dochtercellen. Ongeveer 40% van alle leukemieën valt onder de categorie AML.

De behandeling van patienten met AML bestaat uit chemotherapie, zonodig gevolgd door een stamcel transplantatie. Bij veel patiënten komt de ziekte op een zeker moment weer terug. De kans hierop varieert tussen verschillende AML-typen en wordt sterk bepaald door genetische afwijkingen die aanwezig zijn in de AML cellen. In **hoofdstuk 1** wordt een overzicht gegeven van de processen die deze eigenschappen mede bepalen en een aantal verschillen in die eigenschappen tussen de normale en leukemische hematopoiese.

Geprogrammeerde celdood en AML

Geprogrammeerde celdood of apoptose is één van de processen die de hematopoiese reguleren. Signaaltransductie als gevolg van groeifactorstimulatie heeft niet alleen effect op de groei van, en differentiatie tot verschillende typen bloedcellen, maar verandert tevens overlevingssignalen door genen te beïnvloeden die ingrijpen op het proces van apoptose. Vaak zijn deze signalen op een dusdanige wijze in leukemische cellen verstoord, dat de balans tussen apoptose en overleving in het voordeel van de laatste uitvalt. Chemotherapeutische behandeling is dan ook vaak gebaseerd op het principe van het induceren van apoptose. Echter, in leukemie cellen zijn vaak eiwitten geactiveerd die het proces van apoptose tegen gaan. Hierdoor kunnen AML-cellen chemotherapie resistent zijn of resistentie ontwikkelen.

In dit proefschrift hebben we gekeken naar de effecten van verschillende eiwitten op het proces van apoptose in leukemische cellen. Het eiwit Nuclear Factor of kappa B (NF-κB) is één van de eiwitten die in AML-cellen veelvuldig geactiveerd is, terwijl in normale cellen dit uitsluitend voorkomt na groeifactorstimulatie. In **hoofdstuk 2** laten we zien dat AML- cellen met een geactiveerd NF-κB eiwit, een lagere spontane apoptose hebben dan AML-cellen zonder een geactiveerd NF-κB eiwit. Tevens is de chemotherapeutisch-geïnduceerde apoptose verlaagd in de cellen met een geactiveerd NF-κB eiwit. Door een remmer van het eiwit NF-κB te gebruiken konden we de effectiviteit van chemotherapie verhogen in cellen waarin NF-κB actief is als gevolg van een signaaltransductieroute die RAS, PI3-Kinase en PKB omvat.

De continue activatie van NF-κB in een hoog percentage van de AML-cellen roept de vraag op of NF-κB-activiteit niet alleen betrokken zou kunnen zijn bij chemoresistentie, maar misschien ook bij maligne ontaarding. Verschillende signaaltransductieroutes die betrokken zijn bij leukemische transformatie, leiden tot activatie van NF-κB. Maar of continue NF-κB-activatie voldoende is of dat andere signaaltransductieroutes, alleen of in combinatie met NF-κB, een rol spelen, is niet opgehelderd. In **hoofdstuk 3** zijn de effecten bestudeerd van een geactiveerd NF-κB op de normale hematopoietische stam- en voorloper cel. Overexpressie in $CD34^+$ stam- en progenitorcellen had echter, ondanks de continue activatie, niet tot gevolg dat de cellen zich anders ontwikkelden. Er werden geen verschillen waargenomen in groei, overleving, differentiatie of stamcelactiviteit. Deze experimenten laten zien dat, ondanks een mogelijke rol voor NF-κB in chemoresistentie, continue activatie van NF-κB op zich zelf niet genoeg is om een stam- of progenitorcel te laten afwijken van zijn normale ontwikkelingsprogramma. Dit betekent echter niet dat NF-κB

in samenwerking met andere mutaties niet zou kunnen leiden tot maligne ontaarding. De overexpressie van NF-κB in combinatie met andere eiwitten zou hierin meer inzicht moeten verschaffen.

Een ander eiwit waarvan bekend is dat het een rol speelt in apoptose is p21, mogelijk als gevolg van NF-κB-activatie. In **hoofdstuk 4** is de rol van p21 in AMLs onderzocht. In vergelijking met normale bloedcellen bleek dat p21 zeer sterk en uitsluitend in het cytoplasma tot expressie kwam in 75% van de onderzochte monocytaire AMLs. Juist deze cytoplasmatische variant had een beschreven anti-apoptotische werking. Vergelijking van AMLs met cytoplasmatisch p21 en AMLs zonder p21, bleek een aanmerkelijk verschil in chemotherapeutisch geïnduceerde apoptose op te leveren. Experimenten in cellijnmodellen bevestigden vervolgens dat cytoplasmatische expressie van p21 leidt tot een verminderde apoptose via de ASK1-p38-JNK route. De cytoplasmatische lokalisatie van p21 voorkomt eveneens dat er een groeiarrest optreedt. Beide processen zouden dus kunnen bijdragen aan de voortgaande groei van de leukemische cel.

In **hoofdstuk 5** wordt aangetoond dat de hierboven beschreven ASK1-p38-JNK route niet alleen door p21 geremd kan worden, maar eveneens door het chaperonne eiwit HSP27. Echter, in blasten van AML-patiënten konden we een dergelijke correlatie tussen HSP27 expressie en verlaagde apoptose niet aantonen, wat het gevolg is van een maskerende werking van p21.

Gezamenlijk laten hoofdstuk 2 tot en met 5 zien, dat in AML-cellen er verschillende signaaltransductieroutes geactiveerd zijn. Deze gegevens maken duidelijk dat voor de behandeling van AML-patienten meerdere routes zullen moeten worden geattaqueerd om tot een effectieve behandelingsstrategie te komen. Bovendien lijkt de aanwezigheid van één geactiveerde signaaltransductie route niet verantwoordelijk voor de leukemische transformatie, en dat hiervoor meerdere signalen nodig zijn. Echter het blokkeren of remmen van één van die signaaltransductieroutes kan wel bij dragen aan het anti-leukemische effect van chemotherapie.

Gecontroleerde groei en AML

In normale hematopoiese worden primitieve stam-/progenitorcellen inactief gehouden door remmende groeifactoren, zoals Transforming Growth Factor β (TGF-β), en interacties met stromale cellen in het beenmerg. Dit gebeurt om excessieve groei en daarmee uitputting van het hematopoietische systeem te voorkomen. Verstoring van dergelijke controle mechanismen kan de cyclische activiteit van leukemische cellen verhogen en daarmee een voordeel geven ten opzichte van normale cellen.

Om dit verder te onderzoeken, is in **hoofdstuk 6** gekeken naar het mechanisme waarmee N-Ras^{L61} getransformeerde leukemische cellen ontsnappen aan TGF-β geïnduceerde groeiremming. Daarbij zijn twee leukemische cellijnen met een N-Ras^{L61} mutatie (HL-60 and TF-1) vergeleken met een hematopoietische cellijn zonder Ras mutaties (M1). Het ontbreken van een respons op TGF-β ging gepaard met de afwezigheid van p27 in cellen met de N-Ras^{L61} mutatie. Verschillende experimenten wezen vervolgens op de centrale rol voor p27 in de interactie tussen de Ras en TGF-β signaal transductie routes. Daarnaast bleek het eiwit SKP2, een bekende component van de p27 afbraak machinerie, sterk tot expressie te komen als gevolg van de Ras-mutatie en een rol te spelen in de afbraak van p27. Concluderend tonen deze data aan dat de N-Ras^{L61} mutatie in staat is om de negatieve groeiremming als gevolg van TGF-β-signaaltransductie te blokkeren door middel van afbraak van p27. In ongeveer 10% van de AMLs wordt deze mutatie waargenomen, waarbij onze experimenten een nieuw mechanisme beschrijven, waarmee deze AML-cellen een groeivoordeel zouden kunnen verkrijgen als gevolg van het ontbreken van negatieve controlerende factoren.

Self-renewal, differentiatie en leukemie

Daar waar normale hematopiese een polyklonaal proces is, wat wil zeggen dat meerdere HSCs bijdragen aan de totale hematopoiese, is AML een klonale afwijking. In normale hematopoiese bestaat er een goede balans tussen self-renewal in het stamcelcompartiment en differentiatie richting meer uitgerijpte cellen. In AML bestaat het stamcelcompartiment uit de leukemische stamcel (LSC), die een uitgebreide self-renewal capaciteit, maar een zeer beperkte differentiatie capaciteit bezit. Hierdoor hebben zij een voordeel ten opzichte van normale HSCs.

STAT5 is een transcriptie factor waarvan recentelijk door overexpressie-experimenten is aangetoond dat het een rol speelt in de verhoogde self-renewal van stamcellen. Dit wordt gecombineerd met een afname in expressie van transcriptiefactoren die een rol vervullen in de myeloide differentiatie. Omdat in AML-cellen frequent een geactiveerd STAT5 wordt waargenomen, is in **hoofdstuk 7** onderzocht of afname van STAT5-expressie, doormiddel van RNA-interferentie, effecten heeft op normale- en leukemische-stamcellen. Verlaging van STAT5 met zo'n 80% in CD34^{+} stam- en progenitorcellen zorgde ervoor dat in lange termijn kweken op stromale beenmerg cellen de expansie sterk achterbleef in vergelijking met cellen waarin STAT5 niet verlaagd was. Deze verminderde groei/expansie was niet het gevolg van apoptose of een verhoogde blokkade in celcyclusprogressie. Eveneens bleef de

differentiatiecapaciteit van deze cellen onveranderd. Uit verschillende progenitor- en stamcelkweken bleek dat de STAT5-verlaging aanleiding gaf tot een verminderd aantal celdelingen op het niveau van één enkele cel. Vervolgens hebben we STAT5 ook verlaagd in $CD34^+$ AML-cellen. Ook in deze blasten bleek STAT5-verlaging een verminderde expansie te geven, vergelijkbaar met de vermindering die ook in normale cellen werd gezien. Er werden echter geen effecten op het differentiatieblok waargenomen. De resultaten tonen aan dat zeer onrijpe hematopoietsiche stam- of progenitorcellen afhankelijk zijn van STAT5 voor hun groei. Verder suggereren deze experimenten dat AML-cellen eveneens afhankelijk zijn van STAT5 voor hun groei. Of dit het gevolg is van een verminderde self-renewal zal uit verdere experimenten moeten blijken, maar het feit dat de differentiatie als gevolg van STAT5-verlaging niet veranderde, maar na overexpressie wel, laat zien dat de mate van STAT5-expressie bepalend is voor deze processen.

Onlangs is aangetoond dat de blokkade in myeloide differentiatie na STAT5-overexpressie het gevolg was van verlaging van de CCAAT enhancer binding protein alpha (C/EBPα) expressie. Omdat verschillende studies ook hebben laten zien dat C/EBPα gemuteerd of geïnactiveerd is in AML-cellen, hebben we in **hoofdstuk 8** gekeken naar de effecten van herintroductie van een induceerbare vorm van C/EBPα in $CD34^+$ AML-cellen. In lange-termijnkweken op stromale beenmergcellen wordt aangetoond dat AML-cellen, waarin dit C/EBPα geactiveerd is, onmiddellijk stoppen met groei. Deze groeistop ging gepaard met een gedeeltelijk herstel van myeloide differentiatie. Een interessant detail van deze kweken was dat er bijna geen cellen aangetroffen werden met eigenschappen van de LSCs nadat we C/EBPα geactiveerd hadden, terwijl controle kweken dit wel liet zien. Deze gegevens wijzen erop dat de self-renewal eigenschappen van LSCs beïnvloed worden door C/EBPα. Daarom zijn er vervolgens experimenten uitgevoerd, waarbij gekeken werd of *in vitro* 'getransplanteerde' cellen hieruit naar een nieuwe kweek in staat waren om een nieuwe leukemische kweek te genereren. Daar waar controle AML-cellen inderdaad in staat waren dit tenminste driemaal te doen, bleken de C/EBPα geactiveerde cellen hiertoe, niet in staat. Tot slot zijn enkele genen, die betrokken zijn bij self-renewal en celgroei, bestudeerd. In één AML vonden we inderdaad dat C/EBPα activatie correleerde met een verlaging van self-renewal genen, zoals BMI-1, Meis1 en p21. Echter, andere AMLs lieten dit beeld niet zien, dus verdere experimenten zullen moeten worden uitgevoerd om een verband tussen self-renewal en activiteit van C/EBPα aan te tonen.

In **hoofdstuk 9** wordt tenslotte een samenvatting gegeven van de in dit proefschrift uitgevoerde onderzoeksprojecten. De resultaten worden daarbij in een breder perspectief geplaatst en vergeleken met andere gepubliceerde studies. Tevens wordt aangegeven in welke mate deze nieuwe inzichten naar de toekomst vertaald kunnen worden naar andere en mogelijke betere behandelstrategieën voor AML-patiënten en waar het toekomstig onderzoek zich zou op moeten richten.

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List of abbreviations

ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
AML SL-IC	AML SCID-Leukemia Initiating Cells
Apaf-1	Apoptosis Protease Activating Factor-1
ASK1	Apoptosis Signal Regulating Kinase-1
C/EBPα	CCAAT Enhancer Binding Protein α
CML	Chronic myeloid leukemia
JNK	c-Jun N-terminal Kinase
CAFC	Cobblestone Area Forming Cell
CLP	Common Lymphocyte Progenitor
CMP	Common Myeloid Progenitor
CB	Cord Blood
CDK	Cyclin-Dependent Kinase
CDKi	Cyclin-Dependent Kinase inhibitor
EMSA	Electrophoretic Mobility Shift Assay
Erk	Extracellular signal Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FAB	French-American-British
GMP	Granulocyte Macrophage Progenitors
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
G-CSF	Granulocyte-Stimulating Factor
HSP27	Heat Shock Protein 27
HSC	Hematopoietic Stem Cell
IKK	IκB Kinase
IκB	Inhibitor of NF-κB
IL	Interleukin
ITD	Internal Tandem Duplication
JAK	Janus Kinase
L-CA	Leukemic Cobblestone Area
LSC	Leukemic Stem Cell
LTC	Long Term Culture
LTC-IC	Long-Term Culture-Initiating Cell
LMPP	Lymphoid primed Multipotent Progenitor
M-CSF	Macrophage-Colony Stimulating Factor
MEK1	Map kinase/ERK kinase 1
MkEP	Megakaryocytic/Erythroid progenitor
MAPK	Mitogen-Activated Protein kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MPP	Multipotent Progenitor
MDS	Myelo Dysplastic Syndrome
MPD	Myeloproliferative Disease
NE	Neutrophil Elastase
NRE	NF-κB Responsive Element
NF-κB	Nuclear Factor kappa B
NLS	Nuclear Localization Signal
NPM	Nucleophosmin
PMA	Phorbol 12-Myristate 13-Acetate
PKB	Protein Kinase B
PKC	Protein Kinase C
Q-PCR	Quantitative PCR
Rb	Retinoblastoma
RNAi	RNA interference
STAT	Signal Transducer and Activator of Transcription
SAPK	Stress-Activated Protein Kinase
TGF-β	Transforming Growth Factor-β

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